INTERACTION OF ANTIOXIDANTS WITH DNA

(Studies on the interaction of curcuminoids with DNA)

THESIS

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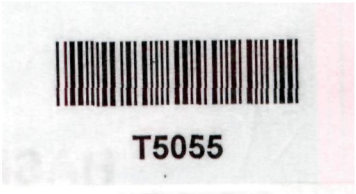

As a token of love

and

deepest affection

to

my family

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CERTIFICATE

This is to certify that the work embodied in this thesis has been carried out by **Mr. Haseeb Ahsan** under my supervision and is suitable for the award of Ph.D. degree in Biochemistry.

(S.M. HADI)
Professor and Chairman

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(HASEEB AHSAN)

CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

LIST OF ABBREVIATIONS

Cu(II)	-	Copper (II) chloride
EDTA	-	Ethylenediaminetetraacetic acid
EtBr	-	Ethidium bromide
µg	-	microgram
µM	-	micromolar
µmol	-	micromoles
mM	-	millimolar
M	-	Molar
nmol	-	nanomoles
Tris-HCl	-	Tris(hydroxymethyl)aminomethane hydrochloride
v/v	-	volume/volume
w/v	-	weight/volume

SUMMARY

Curcumin (diferuloyl methane) obtained from the rhizome of the plant Curcuma longa is a naturally occurring phytochemical and a constituent of the spice-turmeric. It possesses a wide range of pharmacological properties including anti-inflammatory, anti-tumor promoter and anti-oxidant effects. In the first part of the thesis it is shown that in presence of Cu(II), curcumin caused breakage of calf thymus and supercoiled plasmid DNA. In the case of plasmid DNA, the products were relaxed circles with no detectable linear forms. Other metal ions tested such as Mg(II), Mn(II), Ni(II), Ca(II), Fe(II) were ineffective or less effective in the DNA breakage reaction. Cu(I) was shown to be an essential intermediate by using the Cu(I)-specific sequestering reagent, neocuproine. The involvement of active oxygen species such as hydrogen peroxide and singlet oxygen was established by the inhibition of DNA breakage by catalase and azide. Curcumin is also able to directly produce superoxide anion and hydrogen peroxide and in the presence of Cu(II), the hydroxyl radical is generated. Absorption spectra of curcumin in the presence of DNA indicated that a complex is formed between the two. Cu(II) alone is also capable of binding to curcumin. The results are discussed in relation to the established proxidant activities of other known antioxidants.

Curcumin as isolated from turmeric is known to contain demethoxy-curcumin (dmC) and bisdemethoxycurcumin (bdmC) as minor contaminants. It

was of interest to determine the relative DNA degradation activity of curcumin and the demethoxy derivatives. Hence, in the second part of the thesis, I have demonstrated that purified curcumin as well as its two structural analogues namely dmC and bdmC are capable of causing breakage of supercoiled plasmid DNA in the presence of Cu(II). The relative efficiency of DNA cleavage is in the order curcumin > dmC > bdmC. All the three curcuminoids are capable of producing reactive oxygen species- hydroxyl radicals and hydrogen peroxide. The production of hydroxyl radicals is considerably enhanced in the presence of Cu(II) for all the curcuminoids. The three compounds have absorption maxima at around 415 nm and exhibit characteristic spectral changes in the presence of calf thymus DNA and copper ions. I have also studied the antioxidant activity of these compounds in systems generating hydroxyl radicals and singlet oxygen. The three curcuminoids are capable of protecting supercoiled plasmid DNA against hydroxyl radical and singlet oxygen induced cleavage to a similar degree. Finally, it is shown that curcuminoids show preference of binding to AT sequences in DNA, in experiments with restriction endonucleases. From these studies it is indicated that the structural features of curcuminoids that are important for their antioxidant effects are also the ones that render these compounds DNA damaging under appropriate conditions.

INTRODUCTION

INTRODUCTION

In the past decades, there has been much emphasis on the induction of cancer by occupational and industrial pollution factors. There is growing recognition, however, that these may account for only a small fraction of human cancers. It is becoming increasingly clear from epidemiological and laboratory data that diet is an important factor in the etiology of certain cancers.The predominance of certain foods in some countries has been related to the incidence of certain types of cancers in their population. The human diet contains a variety of naturally occurring mutagens and carcinogens (Ames, 1983). Therefore, dietary mutagens have attracted considerable interest in the last decades and a number of studies on dietary practices in relation to cancer have been undertaken. Although, quite a large number of dietary components have been evaluated in microbial and animal test systems, there is still a lack of definitive evidence about their carcinogenecity and mechanism of action in humans. A majority of chemical carcinogens are known to form covalent adducts with DNA and there is a large body of evidence implicating DNA as a critical target in chemically induced cancer (O'Connor, 1981). In order to understand carcinogenesis at the molecular level, it is essential to determine the conformational changes in the target macromolecules and relate these findings to possible aberrations in the functioning of modified macromolecules.

Of late, there has also been an increasing interest in oxygen radicals and lipid peroxidation as a source of damage to DNA, and therefore as promoters of

cancer (Gensler and Bernstein, 1981; Harman, 1981). In addition, a large body of evidence has accumulated over the past decades on the role of oxidative damage to biomolecules in ageing phenomenon, chronic inflammation, autoimmune diseases, in the induction of HIV expression, etc., (Feig and Loeb, 1993; Legrand-Poels et al., 1993; Xanthondakis et al., 1992). Mammalian systems have evolved many defence mechanisms as protection against mutagens and carcinogens. The most important of such mechanisms may be those against oxygen radicals and lipid peroxidation, through both enzymatic and non-enzymatic systems (Sies, 1997). The latter includes antioxidants taken as part of normal diet.

Oxygen Radicals and Cancer

One of the theories of etiology of cancer which is being widely accepted, holds that the major cause is damage to DNA by oxygen radicals and lipid peroxidation (Ames, 1983). Oxygen is not totally innocuous and it has long been known to be toxic to many animals including humans. The deleterious effects of oxygen are said to result from its metabolic reduction to highly reactive and toxic species, known as 'reactive oxygen species' (ROS) (Buechter, 1988). These oxygen free radicals in living organisms include hydroxyl radical ($OH^{\cdot}$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), etc., and can cause tissue damage by reacting with lipids in cellular membranes, nucleotides in DNA and sulphydryl groups in proteins (Knight, 1995). In living cells, ROS are formed continuously as a consequence of both biochemical reactions and external factors and damage from them has been proposed to be

TABLE I

Some Characteristics of Reactive Oxygen Derivatives

	Species	Chemical Symbol	Origin	Properties	Protectors
1.	Superoxide anion	$O_2^{\cdot-}$	$O_2 + e^-$	good reductant, poor oxidant	SOD
2.	Hydroxyl radical	$OH^{\cdot}$	H_2O_2, H_2O	extremely reactive, very low diffusion distance	antioxidants
3.	Hydrogen peroxide	H_2O_2	$O_2^{\cdot-}$, biogeneration	oxidant, high diffusion capability	catalase, glutathione peroxidase
4.	Singlet oxygen	$^1O_2$	$^3O_2$, peroxidation	powerful oxidising agent	Vit C, Vit E, β-carotene

adapted from Yu (1994) and Simic et al., (1989).

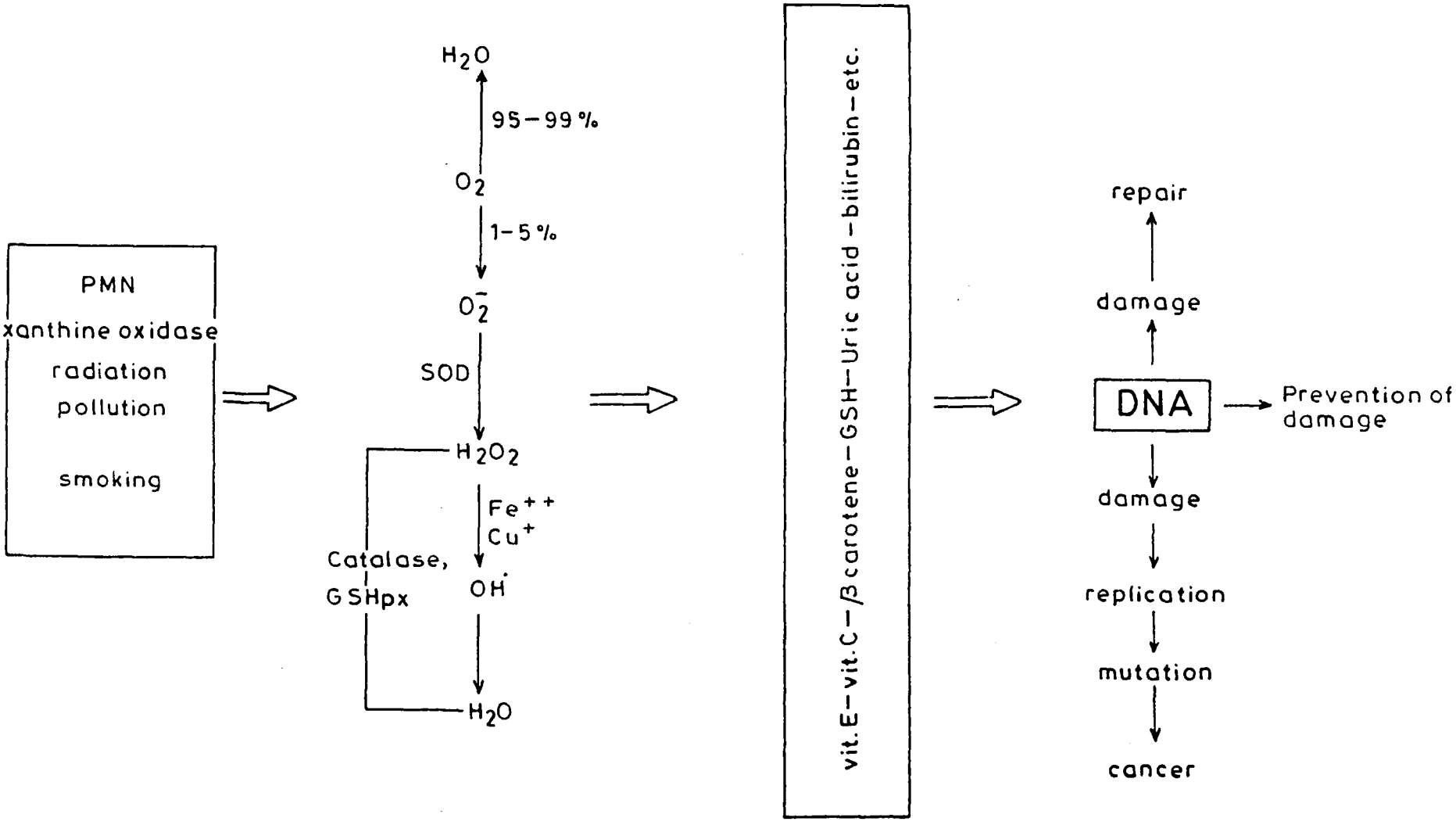
involved in carcinogenesis and age related degenerative diseases. The major source of endogenous oxygen radicals are hydrogen peroxide and superoxide, which are generated as side products of metabolism. In addition, oxygen radicals also arise from phagocytosis after viral and bacterial infection or an inflammatory reaction (Tauber, 1982). The exogenous oxygen radical load is contributed by a variety of environmental agents such as inhaled smoke and polluted air (Nagashima et al., 1995; Frei et al., 1991).

In patients with diseases associated with increased risk of cancer, including Fanconi anemia, chronic hepatitis, cystic fibrosis and various autoimmune diseases, studies indicate an increased rate of oxidative DNA damage or in some instances deficient repair (Brown et al., 1995; Hagen et al., 1994; Shimoda et al., 1994; Takeuchi and Morimoto, 1993). Human studies support the experimentally based notion of oxidative DNA damage as an important mutagenic and carcinogenic factor (Loft and Poulsen, 1996). ROS can damage DNA, and the division of cells with unrepaired or misrepaired damage leads to mutations. The majority of mutations induced by ROS appear to involve modification of guanine, causing G→T transversions (Denissenko et al., 1996; Du et al., 1994; Colapietro et al., 1993; Mass et al., 1993; Higinbotham et al., 1992). If it relates to critical genes such as oncogenes or tumor suppressor genes, initiation/progression can result (Ames et al., 1993). Indeed, these species can act at several steps in multistage carcinogenesis.

It has also been suggested that certain promoters of carcinogenesis act by generation of oxygen radicals, this being a common property of these

substances. Fat and hydrogen peroxide are among the most potent promoters (Welsch and Aylsworth, 1983). Other well-known cancer promoters are lead, calcium, phorbol esters, asbestos and various quinones. Many carcinogens which do not require the action of promoters and are by themselves able to induce carcinogenesis (complete carcinogens), also produce oxygen radicals (Demopoulos et al., 1980). These include nitroso compounds, hydrazines, quinones and polycyclic hydrocarbons. The mechanism of action of promoters involves the expression of recessive genes and an increase in gene copy number through chromosome breaks and creation of hemizygosity (Kinsella, 1982). Promoters also cause modification of prostaglandins which are intimately involved in cell division, differentiation and tumor growth (Fischer et al., 1982). Much of the toxic effect of ionising radiation damage to DNA is also due to the formation of oxygen radicals.

Several enzymes produce superoxide anion during the oxidation of their substrate, for example, xanthine oxidase and peroxidase (Knight, 1995). This radical further accepts an electron from a reducing agent, such as thiols to yield peroxide (H_2O_2). That the superoxide anion actually appears in metabolism is confirmed by the ubiquitous presence of superoxide dismutase. Certain white blood cells generate superoxide deliberately by means of a specialised membrane bound NADPH oxidase and this participates in the killing of microorganisms and tumor cells (Martinez-Cayuela, 1995; Wolff et al., 1986). In view of the catalytic role of enzymes, damage to proteins is also considered important. It has been suggested that primary oxygen radicals produced in cells


PMN
xanthine oxidase
radiation
pollution
smoking
H2O
95–99 %
O2
1–5 %
O2−
SOD
H2O2
Fe++
Cu+
Catalase,
GSHpx
OH·
H2O
vit. E – vit. C – β carotene – GSH – Uric acid – bilirubin – etc.
repair
damage
DNA
Prevention of damage
damage
replication
mutation
cancer

and their secondary lipid intermediates (carbon-centered radicals), modify and fragment proteins. The products are often more susceptible to enzymatic hydrolysis leading to accelerated proteolysis inside and outside the cell (Wolff et al., 1986).

Exposure to ROS and its cellular production are facts of life. ROS can cause oxidative DNA and protein damage, damage to tumor suppressor genes and enhanced expression of proto-oncogenes (Cerutti, 1994; Jackson, 1994), and oxidative stress has been shown to induce malignant transformation of cells in culture (Weitzman and Gordon, 1990). However, the development of human cancer depends on many other factors, including the extent of DNA damage, antioxidant defences, repair enzymes, the efficiency of removal of oxidised nucleosides before they are incorporated into DNA and the cytotoxic effects of ROS in large amounts and their growth promoting effects in small amounts (Burdon et al., 1995).

Metal Ions and Oxidative DNA Damage

There are now rapidly accumulating evidences which strongly suggest that transition metal ions play an important intermediary role in oxygen-mediated injuries to biological macromolecules such as lipids, proteins and DNA. If catalytic metal ions are not present then oxygen free radicals such as superoxide anion and H_2O_2 at physiological concentrations may have limited (if any) damaging effects (Gutteridge, 1994). For example, it is believed that neither $O_2^{\cdot}$ nor H_2O_2 produce DNA strand breaks or modification of DNA bases

under physiological conditions. Most of the toxicity of oxygen and H_2O_2 in vivo is thought to arise from metal ion catalysed production of highly reactive hydroxyl radicals, through the so called 'Fenton reaction' (Aust et al., 1993; Goldstein et al., 1993). The metal ions could be bound to DNA/chromatin or oxidative stress could liberate them from intracellular storage sites with subsequent binding to DNA.

Among the mechanisms proposed to explain the injurious effects of copper and iron is their role in free radical reactions. These redox active metal ions are proposed to be involved in the production of oxygen derived free radicals from relatively low reactive species. As these metal ions are sparingly soluble under physiological conditions, they must attach and form complexes with biomolecules and serve as redox active centres for repeated production of free radicals leading to damage at or near the metal binding site (Chevion et al., 1993; Lesnefsky, 1992). Most iron is safely bound to proteins such as ferritin and transferrin. However, its release from ferritin can be accomplished by reductase enzymes, superoxide or ascorbate (Breen and Murphy, 1995). There is also an increasing dietary intake of iron in the form of Fe^{2+}, which is however well chelated to perform the Fenton reaction. Certain food additives such as ascorbate and phenolic antioxidants may help sustain the Fenton reaction by recycling the iron and when present with Fe^{2+}/EDTA may have negative as well as positive aspects (Aruoma, 1993).

The biochemical basis of copper toxicosis is not clear and presumably multifaceted. Copper is an important constituent of a number of metalloproteins

and metalloenzymes, and the catalytic activity of several enzymes is dependent upon it (Agarwal et al., 1989). The majority of copper metalloenzymes catalyse oxidation-reduction reactions.The toxicity of released copper may involve free radical damage (Halliwell et al., 1992). Copper ions promote lipid peroxidation and catalyse the formation of highly reactive $OH^{\cdot}$ and singlet oxygen from H_2O_2. Mixtures of copper ions and H_2O_2 produce DNA damage-strand breaks and chemical changes in purine and pyrimidine bases, especially conversion of guanine into 8-hydroxydeoxyguanine (Aruoma et al., 1991). Due to its cellular production and diffusion through membranes, H_2O_2 is believed to play a major role in the production of oxidative DNA damage in vivo (Halliwell and Gutteridge, 1989). Copper is found to be more potent than iron in catalysing oxidative damage to DNA (Cai et al., 1995; Tachon, 1989). Excessive tissue copper concentrations may affect the stability of membranes, synthesis and function of proteins and the replication and transcription of DNA (Agarwal et al., 1989; Schilsky et al., 1989). Wilson's disease and genetic hemochromatosis (GH) are conditions in which there is copper or iron overload and reduced stability of DNA (Carmichael et al., 1995; Bacon and Britton, 1990). Copper overload in Wilson's disease is treated with the copper chelator D-penicillinase, which is very successful in expanding the life span of patients with this syndrome (Evans and Johnson, 1982).

Metal ions are also involved in the action of many drugs and xenobiotics. In some cases, metal ions are essential for the expression of the activity of various drugs and toxic agents, while in others they just enhance their effect or

may even provide protection by inhibiting the activity of these compounds (Uddin and Ahmad, 1997). During the last decade or two, studies have focussed on the possible role of copper as a mediator of free radical induced toxicity, including cytotoxicity and genotoxicity (Tkeshelashvili et al., 1991). Some studies also show that the interaction of several xenobiotics with copper results in their metabolism or activation by a redox mechanism (Flowers et al., 1997; Li et al., 1994; Li and Trush, 1993; Swauger, 1991). It is being widely accepted that in the presence of reducing environment or endogenous reductants, copper (both endogenous and exogenous) induces a cyclic Fenton-like reaction which is harmful to macromolecules and cells with metal binding sites being particularly affected (Milne, 1993; Prutz, 1993).

Although a large number of protective mechanisms exist from antioxidants to repair enzymes, but oxidative damage to DNA is abundant in human tissues (Loft and Poulsen, 1996; Demple and Harrison, 1994). Damage to DNA resulting from exposure to ROS may lead to modified bases, abasic sites, single and double strand breaks and DNA-protein crosslinks (Lloyd et al., 1997; Halliwell and Aruoma, 1991). It has been suggested that the cumulative biological effects of oxidative DNA damage over the long human life span leads to ageing and even cancer (Cerutti, 1994; Feig et al., 1994; Frenkel, 1992). Copper and iron are two major transition metals in the biological environment that can catalyse extensive DNA damage in vitro and in vivo (Toyokuni and Sagripanti, 1996, 1992). Since these transition metals are involved in reactions leading to oxygen-associated toxicities, further investigations are required to look at the biological

aspects of transition metal metabolism, which would aid in the development of effective food and drug therapies to prevent oxygen-mediated injuries. Accordingly, a concept of balance between physiological/metabolic functions and the various deleterious effects of ROS and metal ions,apart from others has emerged as the basis for the development of degenerative diseases, including cancer (Loft and Poulsen, 1996; Wiseman and Halliwell, 1996).

Mutagens and Carcinogens in Dietary Plant Material

A large number of toxic chemicals are synthesized by plants, presumably as a defence against a variety of invasive organisms, such as bacteria, fungi and insects (Kapadia, 1982; Clark, 1982). It has been known for many years that plants contain carcinogens, and a number of edible plants have shown experimental carcinogenic activity for several species and various tissues. Wide use of short term tests for detecting mutagens (Stich and San, 1981), and a number of animal cancer tests on plant substances have contributed to the identification of many mutagens and carcinogens in the human diet (Kapadia, 1982).

Ivie et al., (1981) have reported that linear furocoumarins (psoralens), are potent light activated carcinogens and mutagens. Some of the most common phototoxic furocoumarins are psoralen, xanthotoxin and bergapten. Psoralens are potent photosensitizers and highly mutagenic in the presence of activating long wavelength UV light. They readily intercalate into duplex DNA where they

form light induced mono- or diadducts with pyrimidine bases. Psoralen in the presence of light is also effective in producing oxygen radicals (Ya et al., 1982).

Pyrrolizidine alkaloids are naturally occurring carcinogens and are found in some fifty species of plants, which are used as foods or herbal remedies (Schoental, 1982). Several of these alkaloids are hepatotoxic and certain hepatotoxic pyrrolizidine alkaloids are also carcinogenic (Mori et al., 1985). Certain glycoalkaloids found in potato, such as solanine and chaconine, have been reported to be highly toxic as they are strong inhibitors of cholinesterase (Jadhav et al., 1981). Pyrrolizidine alkaloids and other glycoalkaloids can reach levels which can be lethal to humans in potatoes that are diseased or exposed to light (Katsui et al., 1982).

Edible mushrooms contain various hydrazine derivatives in relatively large amounts. Most hydrazines that have been tested have been found to be carcinogenic and mutagenic. The common commercial mushroom, Agaricus bisporus contains about 300 mg of agaritine, the δ-glutamyl derivative of the mutagen, 4-hydroxymethylphenylhydrazine, per 100 gm of mushroom (Toth et al., 1982). Some agaritine is metabolized by the mushroom to a diazonium derivative, which is a potent carcinogen and is also present in the mushroom in smaller amounts. Many hydrazine carcinogens may act by producing oxygen radicals (Hochstein and Jain, 1981).

A number of 1,2-dicarbonyl compounds e.g., maltole, kojic acid, ethylmaltole, diacetyl and glyoxal have been found to be mutagenic in the

Salmonella/Microsome assay. Several compounds in this class are of toxicological interest because they occur in various foods. For example, maltole is a product of carbohydrate dehydration and is present in coffee, soyabeans and baked cereals. Kojic acid is a metabolite of many microorganisms including several fungi used in food production, while diacetyl is an aroma component of butter, beer, coffee, etc. (Fishbein, 1983).

A number of furans, such as 2-methylfuran, dimethylfuran, furfural, 5-methylfurfural and 2-furylmethylketone are found in numerous food products including meat, milk products, tea, coffee. Stich et al., (1981) have reported that these furans induced relatively high frequencies of chromatid breaks and chromatid exchanges when they were exposed to cultured Chinese Hamster Ovary (CHO) cells in the absence of a liver microsomal preparation. The clastogenic doses of many of the furans were relatively high (100-3900 ppm), whereas the concentration in food products was relatively low. However, they also cautioned that the furans are not the only genotoxic chemicals in the complex mixture of heated, roasted or boiled food products, and even if the furans do not pose a serious health hazard by themselves due to their small amounts in most food items, they do contribute significantly to the total genotoxicity of many consumable foods and beverages.

Cyclopropenoid fatty acids present in cotton seed and other oils, have been reported to be carcinogenic and mitogenic having various toxic effects in farm animals. Among these, sterculic acid and malvalic acid are widespread in the human diet. They are also potentiaters of carcinogenicity of aflatoxins

(Hendricks et al., 1980). Another major toxin in cotton seed is gossypol. Gossypol causes male sterility through formation of abnormal sperm. It is a potent initiator and also promoter of carcinogenesis in mouse skin (Haroz and Thomassan, 1980) and is carcinogenic as well (Xue, 1980). Gossypol has been tested as a possible male contraceptive, as it is inexpensive and causes sterility during use. Its mode of action as a spermicide is presumably through the production of oxygen radicals.

A number of quinones and their phenolic precursors are found in the human diet and have been shown to be mutagens (Levin et al., 1982). Quinones are quite toxic as they can act as electrophiles or accept a single electron to yield the semiquinone radicals which can react directly with DNA or generate superoxide radicals (Morimoto et al., 1983). Many dietary phenols can autoxidize to quinones, generating hydrogen peroxide at the same time (The amount of these phenols in human diet are appreciable). Catechol which is mainly derived from metabolism of plant substances is a potent promoter of carcinogenesis and an inducer of DNA damage (Carmella et al., 1982).

In addition, there are many other dietary compounds which have been shown to be mutagenic and carcinogenic in various test systems. Allyloisothiocyanate, a major flavour ingredient of mustard oil, is one of the main toxins of mustard seeds and has been shown to be a carcinogen in rats (Dunnick et al., 1982). Phorbol esters are potent promoters of carcinogenesis and cause nasopharyngeal and esophageal cancers (Hecker, 1981). Nitrosoamines and other nitroso compounds formed from nitrate and nitrites in

food have been directly related to the incidence of stomach and esophageal cancer. Nitrates are present in large amounts in spinach, radish, lettuce and beans (Magee, 1982). A variety of carcinogens and mutagens are present in mold contaminated food grains, nuts and fruits. Some of these, such as various aflatoxins, are among the most potent carcinogens and mutagens known (Tazima, 1982).

Food Additives

Sodium nitrite is used as a preservative in meat, fish and cheese. A possible formation of nitrosoamines from amines, present in or derived from the diet, occurs by reaction with nitrous acid at acidic pH. A high concentration of hydrogen ions in the human stomach (gastric juice attains a pH of around 1.0) gives rise to the nitrosyl cation NO^+, which is a highly reactive nitrosylating agent. Nitrous acid itself is a known mutagen for various bacterial and fungal cells. Its mutagenicity is presumably related to the deamination of adenine and cytosine. Sodium bisulphite is used as a bacterial inhibitor in a variety of beverages and as a preservative in canned fruits and vegetables. The bisulphite anion reacts rather specifically with uracil and cytosine, within single stranded regions of DNA and RNA. It is also mutagenic to bacteria and bacteriophages (Singer, 1983). Alkali salts of EDTA are widely used as sequesterants in various foods. They are useful as antioxidants due to their property of forming poorly dissociable chelate complexes with trace quantity of metal ions such as copper and iron in fats and oils. EDTA has been shown to induce chromosome aberrations and breakage in drosophila and various plant species.

Anticarcinogens

If oxygen radicals play a major role in damage to biomolecules, especially DNA, defence against these agents is obviously of great importance. The enzymes that protect cells from oxidative damage are superoxide dismutase, glutathione peroxidase, diaphorase and glutathione transferases (Lind et al., 1982; Warholm et al., 1981). In addition to these enzymes, some small molecules in the human diet act as antioxidative agents and presumably have an anticarcinogenic effect. Epidemiological evidence from cross-cultural and case control studies almost point unanimously to reduced risk for cancer, particularly in the upper gastrointestinal tract and airways, associated with a diet rich in antioxidants and/or a high content of antioxidants in human plasma (Ames et al., 1995; Block, 1992; Block et al., 1992). While a higher cellular antioxidant capacity tends to protect DNA from oxidative damage and related mutagenesis, antioxidant activity may also protect initiated cells from ROS mediated killing (Halder et al., 1994).

Antioxidant defence against free radical damage includes vitamin E, vitamin C, β-carotene, glutathione, uric acid, bilirubin, several metalloenzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase and proteins such as ceruloplasmin (Yu, 1994). The extent of damage is a result of the balance between free radicals generated and the antioxidant protective defence system (Machlin and Bendich, 1987). We also obtain several antioxidants from the diet. The consumption of fruits, grains and vegetables, which are the main source of these antioxidants, is of importance in protecting

against oxidative damage and resulting diseases (Gey, 1995; Diplock, 1994; Halliwell, 1994). Intake of fresh fruits and vegetables seems to be inversely correlated with cancer of stomach, pancreas, oral cavity and esophagus. In addition to antioxidants, fruits and vegetables contain many vital micronutrients that may be protective (Bertram, 1993; Krinsky, 1993).

Tocopherol (vitamin E) is an important trap of oxygen radicals in membranes and has been shown to decrease the carcinogenic effect of quinones, adriamycin and daunomycin which are toxic because of free radical generation (Ames, 1983). Protective effect of tocopherols against radiation induced DNA damage and dimethylhydrazine induced carcinogenesis have also been observed (Beckman et al., 1982). β-carotene is a potent antioxidant present in the diet and is important in protecting lipid membranes against oxidation. Singlet oxygen is a highly reactive form of oxygen which is mutagenic and is generated by the pigment mediated transfer of light energy to oxygen. Carotenoids are free radical traps and are remarkably efficient as quenchers of singlet oxygen (Di Mascio, 1990). β-carotene and similar polyprenes are also the main defence in plants against singlet oxygen generated as a by product of the interaction of light and chlorophyll (Krinsky and Deneke, 1982). Carotenoids have been shown to be anticarcinogenic in rodents and may also have a similar effect in humans (Mathews-Roth, 1982). Glutathione transferases are a major defence against oxidative and alkylating carcinogens (Warholm et al., 1981). Selenium, which is present in the active site of glutathione peroxidase, is another important dietary anticarcinogen. Glutathione peroxidase is essential for

destroying lipid hydroperoxides and endogenous hydrogen peroxide and therefore, helps to prevent oxygen radical induced lipid peroxidation (Flohe, 1982). Some other dietary antioxidants include ascorbic acid and lycopene (Machlin and Bendich, 1987). The former has been shown to be anticarcinogenic in rodents treated with UV light and benzo(α)pyrene (Hartman, 1982). Uric acid is present at high concentrations in the blood of humans and is a strong antioxidant (Halliwell and Gutteridge, 1990; Ames et al., 1981). A low uric acid level has been considered a risk factor in cigarette caused lung cancer; however, too high levels may cause gout.

In addition, edible plants contain a variety of substances such as phenols that have been reported to inhibit or enhance carcinogenesis and mutagenesis in experimental animals (Ames, 1983). The inhibitory action of such compounds may be due to the induction of cytochrome P-450 and other metabolic enzymes (Boyd et al., 1982). A high dose of such compounds may even lead to deleterious side effects. The differences in cancer rates of various populations are generally considered to be due to environmental and life style factors such as smoking, dietary carcinogens and promoters. However, these differences may also be due to insufficient amounts of anticarcinogens and other protective factors in the diet.

According to Doll and Peto (1981), there are five possible ways whereby diet may affect the incidence of cancer : i) ingestion of powerful direct acting carcinogens or their precursors, ii) affecting the formation of carcinogens in the

body, iii) affecting transport, activation or deactivation of carcinogens, iv) affecting promotion of cells that are already initiated and v) overnutrition. In summary, most studies involving cancerous tissue or other samples from patients with malignant diseases or diseases associated with an increased risk of cancer show signs of an increased rate of oxidative DNA modification or in some instances deficient repair. This supports the experimentally based notion of oxidative DNA damage as an important mutagenic and apparently carcinogenic factor. However, the proof of a causal relationship in humans is still lacking. In future, the use of biomarkers may provide this evidence and allow further investigation of the qualitative and quantitative importance of oxidative DNA modification and carcinogenesis in humans and also elucidate possible preventive measures (Loft and Poulsen, 1996).

Antioxidant Role of Curcumin

Antioxidants are potent antimutagenic agents and inhibitors of carcinogenic compounds that bind to DNA. A class of antioxidants is provided by a large number of polyphenolic plant agents that are being used in folk medicine and food. Protection against potential carcinogenic substances is probably one of the most challanging aspects related to these phenolic antioxidants. Their protective effect may be due to the removal of oxygen radicals that are ultimately involved in DNA damage (Kahl, 1991).

An important constituent of the Indian diet is turmeric, rhizome of the plant Curcuma longa, commonly used as a spice in cooking and also as a food preservative and colouring agent. Curcumin (diferuloyl methane), a yellow

orange compound which is the active constituent or the major pigment of this rhizome has been identified as a natural antioxidant. Curcumin is used to colour cheese and butter, in cosmetic formulations and in some pharmaceutical preparations (Govindarajan, 1980). It has been approved for use as a colourant and preservative in food processing (WHO Food Additives, 1975) and is also used as a colouring principle in drugs. Toxicologically, curcumin is relatively inert. It does not appear to be toxic to animals or humans even at high dosage (Deodhar et al., 1980; Shankar et al., 1980). The pharmacological safety of curcumin can be assessed by its consumption for centuries by people upto 100 mg/day in some countries (Ammon and Wahl, 1991).

Curcumin exhibits a variety of toxicological, pharmacological and photochemical activities, including phototoxicity to bacteria, especially the Gram positive species (Dahl et al., 1989) and to rat basophilic leukemia cells in culture (Dahl et al., 1994). The anticarcinogenic properties of curcumin in animals has been demonstrated by its inhibition of tumor initiation induced by benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene (DMBA) and tumor promotion induced by phorbol esters in mouse skin (Huang et al., 1992a). It is believed that it possibly prevents tumorigenesis by modulating arachidonic acid metabolism (Huang et al., 1992b, 1991). A recent study demonstrated that it inhibits azoxymethane-induced colon carcinogenesis (Rao et al., 1995). It has also been shown that curcumin prevents the TPA-induced expression of c-fos, c-jun and c-myc proto-oncogene mRNAs (Kakar and Roy, 1994). Curcumin has been found to inhibit the formation of the potentially mutagenic DNA-adduct of 8-

hydroxydeoxyguanine in mouse skin (Shih and Lin, 1993). Curcumin is being developed in the treatment of arthritic patients and such studies are in phase II of clinical trials (Srimal, 1993; Asthana, 1992-93). It also possesses anti-inflammatory (Satoskar et al., 1986; Rao et al., 1982) and antioxidant properties (Toda et al., 1988, 1985).

Curcumin has a dual role in oxygen radical reactions. It can act as a scavanger of hydroxyl radicals or catalyse their formation from hydrogen peroxide in vitro, depending upon the experimental conditions (Tonnensen and Greenhill, 1992; Kunchandy and Rao, 1990; Tonnensen, 1989). Curcumin is capable of photoproducing active oxygen species - $^1O_2$ and O_2^- (upon irradiation with visible light). Illuminated curcumin initiates multiple photochemical pathways, including photogeneration of $^1O_2$, photoreduction of O_2 to O_2^- and H_2O_2 and production of carbon centered radicals that may subsequently react with O_2 (Dahl et al., 1994). Studies have shown that it is a good scavanger of hydroxyl radicals at high concentration, but at low concentration it activated the Fenton system to generate an increased amount of hydroxyl radicals. It is also a potent scavanger of superoxide radicals and this property may be responsible for its good anti-inflammatory activity (Kunchandy and Rao, 1990). Curcumin may inhibit the promotion of tumors by functioning as an $OH^·$ scavanger (Shih and Lin, 1993).

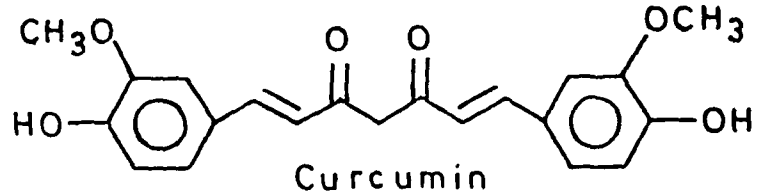
Anticarcinogenic and other therapeutic activities of natural compounds are correlated with their ability to protect biomolecules against reactive oxygens. Curcumin has been shown to be responsible for the protection of DNA from free

radical induced damage (Donatus et al., 1990), hemoglobin from nitrite induced oxidation (Unnikrishnan and Rao, 1992), RBC from primaquine induced oxidative damage (Tonnensen et al., 1994), hepatocytes from various toxins (Shalini and Srinivas, 1990). It also inhibits lipid peroxidation. Curcumin enclosed in liposomes can be used as a drug delivery system (Tonnensen et al., 1993). The free radical oxidation of lipids in foods is a matter of concern for food manufacturers as the degradation of fats gives rise to unpleasant end products. The use of antioxidants in food packaging minimizes such deterioration. Natural foods of plant origin contain components that may have antioxidative properties. So, there is an increasing interest in the use of natural antioxidants from plants in the preservation of food (Aruoma, 1993). Thus, various studies support the role of curcumin as an effective radical quencher and antioxidant which helps in controlling oxygen radical mediated pathologies.

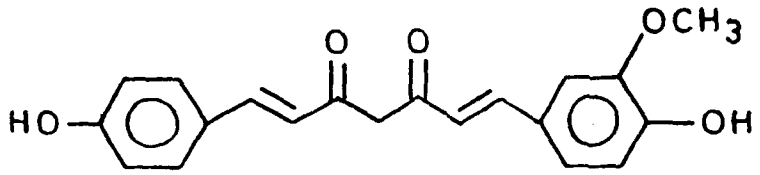
Naturally Occurring Derivatives of Curcumin

The rhizome of Curcuma longa contains in addition to the major component curcumin, two closely related derivatives - demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC). The structure of these compounds is shown in figure 2. Subramanian et al.,(1994) have shown that curcumin and its derivatives have significant abilities to protect plasmid DNA against strand breaks induced by singlet oxygen. Curcumin was found to be the most effective inhibitor of DNA cleavage followed by dmC and bdmC. While according to Sreejayan and Rao (1994), these three curcuminoids were equally potent as inhibitors of iron-mediated lipid peroxidation in rat brain homogenates and liver

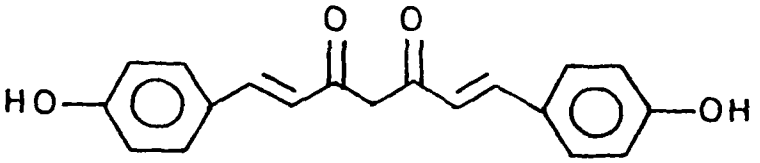
microsomes. More recently, these natural curcuminoids were also studied for their ability to scavange superoxide anions (Sreejayan and Rao, 1996). It was found out that curcumin is the most potent scavanger of $O_2^{\cdot}$ radicals followed by dmC and bdmC. Curcuminoids appear to be the major chemopreventive principles in turmeric and the various mentioned properties may partly help in explaining their protective effects observed in model systems.



Curcumin



Demethoxycurcumin



Bisdemethoxycurcumin

Figure 2 : Structure of curcuminoids

Previous studies from this laboratory have demonstrated that flavonoids cause strand scission in DNA in vitro, in the presence of Cu(II) and molecular oxygen (Rahman et al., 1992). In addition, the natural physiological antioxidant, uric acid has also been shown to cause strand breakage in DNA (Shamsi and Hadi, 1995).

Oxygen radicals have been suggested to be involved in the action of a number of DNA damaging drugs. Curcumin has significant abilities to protect DNA against singlet oxygen ($^1O_2$), a reactive oxygen species with potentially known genotoxic/mutagenic properties. The protective ability of curcumin is reported to be more than that of well known biological antioxidants, e.g. lipoate, α-tocopherol, β-carotene (Subramanian et al., 1994). In view of the previous findings (Jain et al., 1996; Shamsi and Hadi, 1995; Bhat and Hadi, 1994) which suggest that several of the biological antioxidants are themselves capable of DNA damage in the presence of transition metals, I have investigated the effect of curcumin on DNA in the presence of Cu(II).

As mentioned in the previous section, most of the studies with curcumin have been carried out using commercially obtained curcumin or curcumin purified from such preparations. However, commercial grade curcumin is a mixture of about 77% curcumin, 17% demethoxycurcumin (dmC), 3% bisdemethoxy-curcumin (bdmC) and other derivatives (Huang et al., 1995). Huang et al., (1995) have compared the effect of these compounds on 12-0-tetradecanoylphorbol-13-acetate (TPA) induced tumor promotion. Commercial curcumin, pure curcumin and dmC had an equally potent inhibitory effect on

TPA-induced tumor promotion in mouse skin, while bdmC was less active. It has also been shown that all the three components i.e., curcumin, dmC and bdmC brought about a dose dependent inhibition of DNA adduct formation by benzo(α)pyrene. Curcumin was the most effective while bdmC was the least (Deshpande and Maru, 1995).

In order to explore the structure-activity relationship in the curcumin-Cu(II) mediated DNA degradation reaction, I have also compared this activity of these demethoxy derivatives. Further, the inhibitory effect of these three compounds on DNA cleavage by photoexcited riboflavin, Fe-EDTA/H_2O_2 system and radical generation was also examined.

EXPERIMENTAL

MATERIALS

Chemicals and Biochemicals used for the present study were obtained from the following sources.

Chemicals	Source
Agarose	Gibco BRL, USA
Calf thymus DNA (type I, sodium salt)	Sigma Chem. Co., USA
Catalase	Sigma Chem. Co., USA
Cupric chloride	S.D. Fine Chem., India
Curcumin	Aldrich Chem. Co., UK
Ethidium bromide	Sigma Chem. Co., USA
Ethylenediaminetetraacetic acid (disodium salt)	Qualigens Fine Chem., India
Lambda phage DNA	Isolated and purified according to Sambrook et al., (1989)
Neocuproine hydrochloride	Sisco Res. Lab., India
Nitroblue tetrazolium (NBT)	Sisco Res. Lab., India
Nuclease S_1 (A. oryzae)	Sigma Chem. Co., USA
p-nitrosodimethylaniline (pRNO)	Aldrich Chem. Co., USA
Restriction enzymes	Bangalore Genei, India
Riboflavin	Sigma Chem. Co., USA
Supercoiled plasmid DNA (pBR322)	Prepared according to the method of Sambrook et al., (1989)
Superoxide dismutase (Bovine erythrocyte)	Sigma Chem. Co., USA

Titanium dioxide	Loba Chemie, India
Tris (hydroxy methyl)amino methane	E. Merck (India) Ltd.

All the other chemicals were of analytical grade and highest purity available.

METHODS

Assay of S_1 Nuclease Hydrolysis

The enzyme assay was done by estimating the acid soluble nucleotides released from DNA as a result of enzymatic digestion. The reaction mixture (0.5 ml) contained 10 mM Tris-HCl (pH 7.5) and 500 µg of calf thymus DNA (native, denatured and treated). The reaction was started by the addition of Cu(II). All solutions were autoclaved before use.

Nuclease S_1 digestion of the mixture was carried out in a total volume of 1 ml by adding sodium acetate buffer (0.1 M, pH 4.5), 1 mM $ZnSO_4$ and 20-30 units of S_1 enzyme. Reaction mixtures were incubated for 2 hours at 37°C. The reaction was stopped by adding 0.2 ml of bovine serum albumin (10 mg/ml) and 1 ml of 14% perchloric acid (cold). The tubes were transferred to 4°C for at least an hour before centrifugation, to remove the undigested DNA. Nucleotides were determined in the supernatant using the method of Schneider (1957). To a 1 ml aliquot, 2 ml diphenylamine reagent (freshly prepared by dissolving 1 gm of diphenylamine in 100 ml glacial acetic acid and 2.75 ml of conc. H_2SO_4) was added. The tubes were heated in a boiling waterbath for 20 min. The intensity of blue colour was read at 600 nm, after cooling.

Treatment of Supercoiled Plasmid pBR322 DNA with Curcuminoids and Cu(II)

Reaction mixtures (30 µl) contained 10 mM Tris-HCl (pH 7.5) and other components as described in the 'Legends'. All the solutions were autoclaved before use.

After incubation, 10 µl of a solution containing 40 mM EDTA, 0.05% bromophenol blue tracking dye and 50% glycerol (v/v) was added to stop the reaction. The reaction mixtures were subjected to electrophoresis in Tris-acetate EDTA (TAE) buffer (pH 8.0) in a 1-1.4% submarine agarose gel. The gel was stained with ethidium bromide (0.5-1 µg/ml), viewed and photographed on a transilluminator.

Detection of Superoxide Anion

Superoxide anion was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described by Nakayama et al., (1983). A typical assay mixture contains 50 mM sodium phosphate buffer (pH 8.0), 70 µM NBT, 0.1 mM EDTA and 0.06% triton X-100 in a total volume of 3 ml. The reaction was started by the addition of NBT. Immediately after mixing, the absorbance was read at 560 nm against a blank which did not contain curcumin. To confirm the formation of $O_2^{\cdot}$, SOD was also added to the reaction mixture before adding curcumin.

Estimation of Hydroxyl Radical

Hydroxyl radical production was determined by the aromatic hydroxylation method. This assay is based on the ability of $OH^{\cdot}$ radical to hydroxylate aromatic rings at almost diffusion controlled rates and the measurement of hydroxylated products by simple colourimetric method using salicylate (2-hydroxybenzoate) as a detector molecule (Richmond et al., 1981). The reaction mixture (2 ml) contains 2 mM salicylate, 0.1 mM EDTA, 0.1 mM Cu(II) and 50 mM KOH-KH_2PO_4 buffer (pH 8.0). Reaction was started by the addition of curcuminoids and incubated at room temperature for half an hour. The reaction was stopped by adding 80 µl of 11.6 M HCl, 0.5 gm NaCl and 4 ml of chilled diethyl ether. The contents were mixed by vortexing for a minute. Next, 3 ml of the upper ether layer was extracted and evaporated to dryness at around 50-60°C. The dried residue was dissolved in 0.25 ml of cold distilled water and the following added in sequence - 0.125 ml 10% (w/v) TCA (in 0.5 M HCl), 0.25 ml of 10% (w/v) sodium tungstate (in water) and 0.25 ml of 0.5% (w/v) $NaNO_2$ (sodium nitrite, freshly prepared). After standing for 5 min, 0.5 M KOH was added and the absorbance read after a minute, at 510 nm.

Assay of Hydrogen Peroxide Production

The production of H_2O_2 was assayed by the method of Nakayama et al., (1983). Titanium sulphate solution was prepared from titanium dioxide and concentrated sulphuric acid (Snell and Snell, 1949).

A 2 ml sample containing different amount of curcuminoids were mixed with 2 ml sodium phosphate buffer (50 mM, pH 7.2) and incubated at 37°C for one hour. An aliquot (2 ml) of the mixture was added to 2 ml of $Ti(SO_4)$ solution. Absorbance was measured at 410 nm against a blank which did not contain $Ti(SO)_4$ but contains 2 ml sulphuric acid. In order to confirm that the colour change was due to the generation of H_2O_2, 0.4 ml catalase (1 mg/ml) was added in a separate reaction before incubation at 37°C.

Spectroscopy

The absorption spectra were obtained by using a Beckman DU-40 spectrophotometer fitted with a plotter. The absorption data were obtained in a 1 cm path-length cell. All spectroscopic work was carried out at ambient temperatures.

Singlet Oxygen Monitoring And Quenching

Singlet oxygen was measured in aqueous solution by the method of Kraljic and El Mohsni (1978). The ability of riboflavin to form $^1O_2$ was determined by monitoring the bleaching of p-nitrosodimethyl aniline (pRNO) (Joshi, 1985). Histidine was added to the pRNO solution as a selective acceptor of $^1O_2$. pRNO solution was prepared in 0.01 M phosphate buffer. Singlet oxygen formed a transannular peroxide intermediate complex with histidine leading to the bleaching of pRNO, which was then measured spectrophotometrically at 440 nm. Quenching studies with curcuminoids were carried out in 0.15 M phosphate buffer, pH 8.0.

Treatment of λ-DNA With Curcuminoids And Restriction Enzyme Digestion

Lambda phage DNA (1-2 μg) was incubated in a total volume of 30 μl in 10 mM Tris-HCl (pH 7.5) with curcumin and its derivatives. The reaction mixtures were incubated at room temperature for 1 hour, after which they were dialysed against Tris-HCl buffer (10 mM) using 0.025 μM pore size Millipore filters to remove unreacted drugs. Samples were then digested with 2-3 units of various restriction enzymes. EcoRI* activity of restriction endonuclease EcoRI was obtained by using the conditions of low ionic strength, high pH and a high enzyme concentration, as described by Polisky et al., (1975). The reaction was stopped by adding 1 volume of a solution containing 0.2% SDS, 20% sucrose and 0.1% bromophenol blue. The mixtures were electrophoresed on a 1% agarose gel, stained with ethidium bromide (0.5 μg/ml) and photographed over the transilluminator.

Further Purification of Commercially Obtained Curcumin

Curcumin procured from Aldrich Chemical Company, was adsorbed on silica gel (60-120 mesh) and transferred to a column of silica gel. The column was eluted with dichloromethane-acetic acid (95 : 5). Different fractions were collected which were further purified by preparative thin layer chromatography on SiO_2 ($CHCl_3$: EtOH, 25 : 1) as C_1, C_2 and C_3.

C_1 : Eluted with acetone and crystallized from alcohol as orange-yellow needles, highly soluble in acetone, m.p. 182-183°, Rf 0.28 (benzene-ethyl acetate, 7 : 3), gave brown colour in UV light with alcoholic $FeCl_3$. It was comparable with curcumin (Roughley and Whiting, 1973; Janaki and Bose, 1967).

C_2 : Eluted with acetone and crystallized in acetone-hexane as orange crystals, soluble in acetone, m.p. 168-169°, Rf 0.35 (SiO_2, benzene-ethylacetate, 7:3), brown under UV light and gave brown colour with alcoholic $FeCl_3$. It was found to be comparable with demethoxy curcumin (Roughley and Whiting, 1973; Janaki and Bose, 1967).

C_3 : Eluted with acetone and methanol and crystallized from methanol as orange powder, soluble in methanol, m.p. 216-217°, Rf 0.48 (SiO_2, benzene-ethylacetate, 7 : 3), brown in UV and gave brown colour with alcoholic $FeCl_3$. It was found to be comparable with bis-demethoxy curcumin (Roughley and Whiting, 1973; Janaki and Bose, 1967).

RESULTS
(PART I)

RESULTS (Part I)

Breakage of calf thymus DNA by curcumin and Cu(II)

Figure 3 shows the S_1–nuclease hydrolysis of native calf thymus DNA incubated with increasing concentrations of curcumin in the presence of Cu(II). There was a gradual increase in DNA degradation with increasing curcumin concentration. Control experiments showed that heat denatured DNA underwent 100% hydrolysis whereas only about 8% of the native DNA was hydrolysed following treatment with S_1-nuclease at 37°C (data not shown). Curcumin does not significantly inhibit the S_1-nuclease activity at the concentrations tested. In the presence of Cu(II) (0.2 mM), curcumin generated a dose dependent increase in S_1-sensitive sites in calf thymus DNA and maximum hydrolysis was observed following treatment with 200 μM curcumin.

The strand scission in DNA induced by curcumin and Cu(II) is also dependent upon Cu(II) concentration (Fig. 4). A similar progressive increase in DNA hydrolysis was observed when calf thymus DNA was incubated with 50 μM curcumin and increasing concentrations of Cu(II).

Cleavage of plasmid DNA by curcumin and Cu(II)

Supercoiled plasmid pBR322 DNA was examined as a substrate as the relaxation of such a molecule is a sensitive test for just one nick per molecule that results in its conversion to open circular form. Curcumin converted

Figure 3 : **Degradation of calf thymus DNA as a function of increasing curcumin concentration in the presence of Cu(II) measured by the degree of S_1-nuclease digestion.**

DNA was incubated with increasing concentrations of curcumin in the presence of 0.2 mM Cu(II), overnight at room temperature.

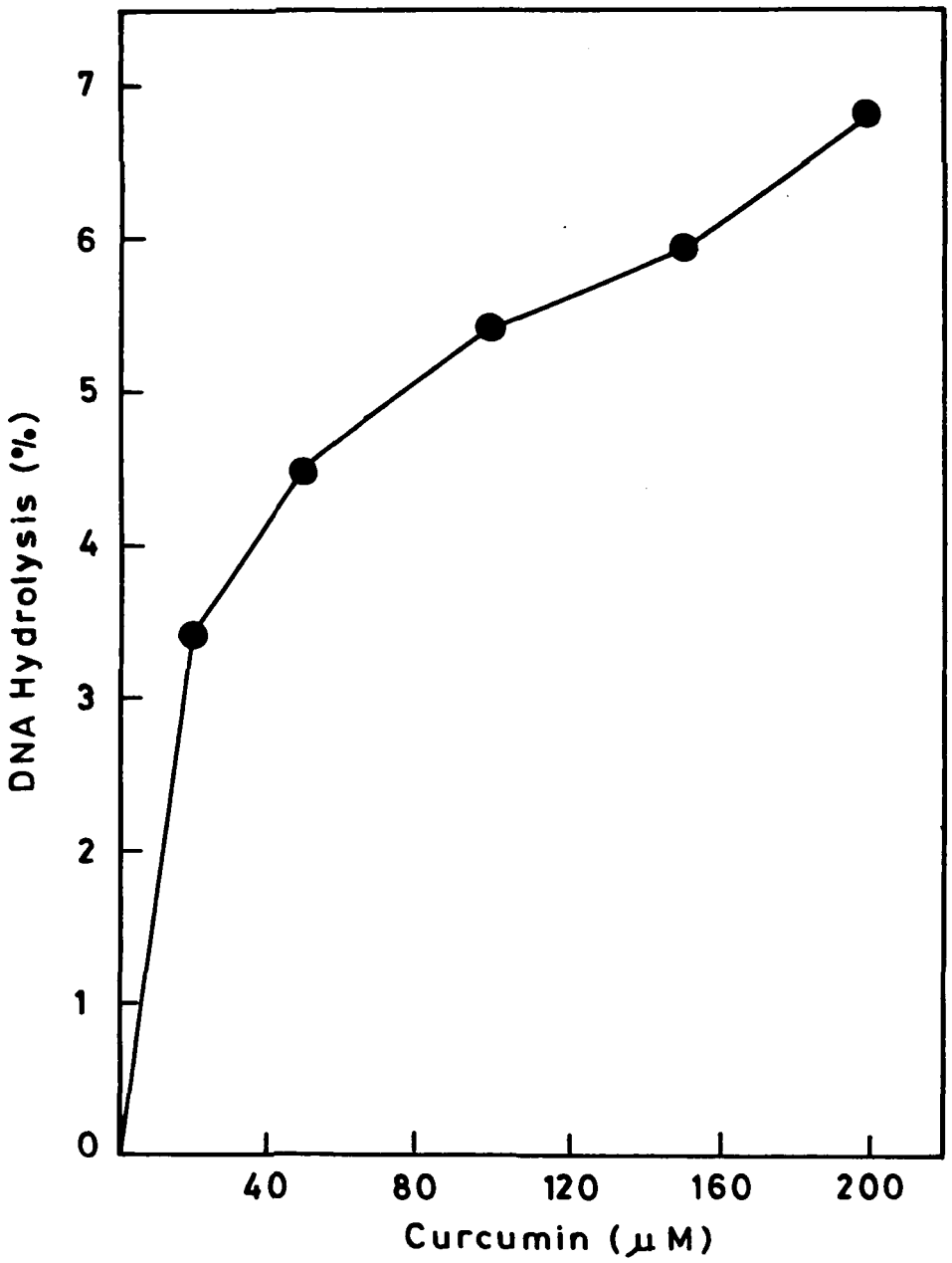
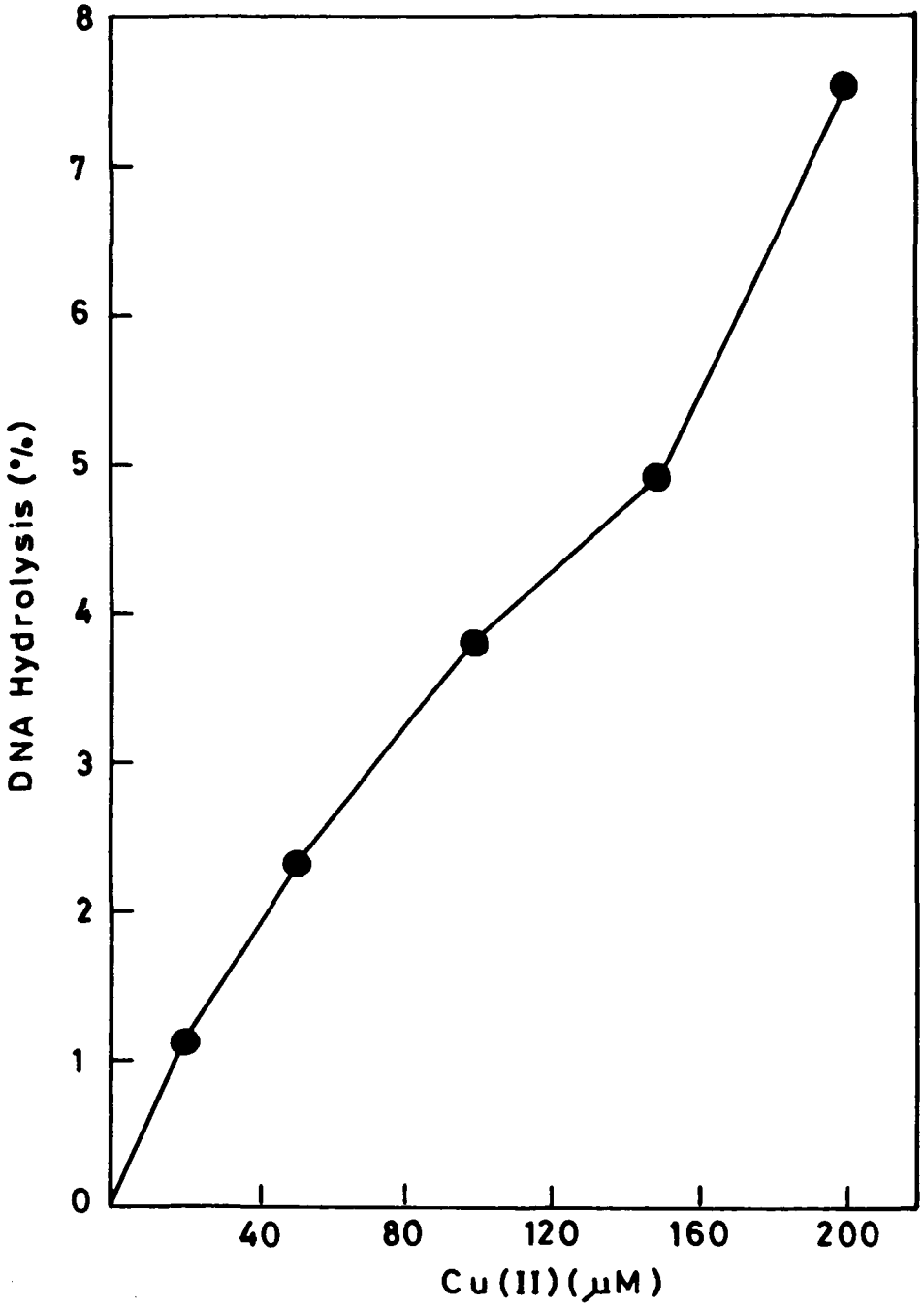

DNA Hydrolysis (%)
0
1
2
3
4
5
6
7
40
80
120
160
200
Curcumin (μM)

Figure 4 : **Degradation of calf thymus DNA as a function of increasing Cu(II) concentration in the presence of curcumin measured by the degree of S_1-nuclease digestion.**

DNA was incubated with increasing concentrations of Cu(II) in the presence of 50 μM curcumin,overnight at room temperature.


DNA Hydrolysis (%)
0
1
2
3
4
5
6
7
8
40
80
120
160
200
Cu (II) (μM)

supercoiled plasmid DNA to relaxed open circles in the presence of Cu(II). Figure 5 shows the effect of increasing curcumin concentration on supercoiled plasmid pBR322 DNA in presence of a fixed concentration of Cu(II). It may be noted that relatively higher concentrations of curcumin (0.4 mM and above) result in an inhibition of strand breakage activity. Similarly with increasing concentrations of Cu(II), a dose dependent cleavage was observed at a curcumin concentration of 100 μM (Fig. 6). However, at a higher concentration of Cu(II), the reaction was inhibited. Figure 7 shows the time dependent conversion of supercoiled (form I) to open circular (form II) DNA mediated by curcumin and Cu(II). A progressive increase in the amount of form II DNA with a concomitant decrease in form I DNA is seen.

Effect of transition metal ions and incubation in light and dark

Figure 8 shows the effect of several metal ions on the degradation of plasmid DNA in presence of curcumin. It is seen that only Cu(II) and to some extent Fe(II) complemented curcumin in the DNA breakage reaction. However, no breakage of supercoiled plasmid DNA occurs if the reaction is performed in both light and dark conditions but in the absence of Cu(II) (Fig. 9).

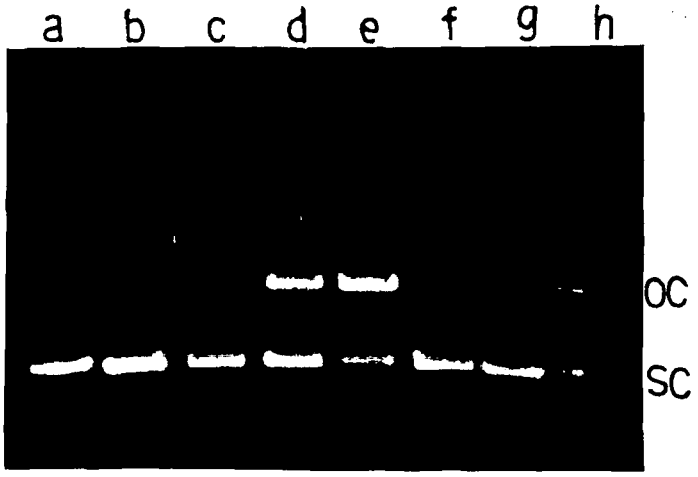
Inhibition of curcumin-Cu(II) induced DNA breakage by neocuproine

It was of interest to determine whether the production of Cu(I) from Cu(II) during curcumin-Cu(II) interaction was necessary for DNA breakage. For this purpose, a Cu(I) specific chelating agent, neocuproine was added to the

Figure 5 : **Effect of increasing curcumin concentration on supercoiled plasmid DNA in the presence of Cu(II).**

Reaction mixtures containing 0.75 μg plasmid pBR322 DNA, 0.1 mM Cu(II) and increasing concentrations of curcumin were incubated for 2 hours at 37°C (OC - open circular DNA, SC - super coiled DNA).

Lane (a) DNA alone, (b) DNA + Cu(II), (c) DNA + curcumin (0.8 mM), (d) DNA + Cu(II) + curcumin (0.05 mM), (e) DNA + Cu(II) + curcumin (0.1 mM), (f) DNA + Cu(II) + curcumin (0.2 mM), (g) DNA + Cu(II) + curcumin (0.4 mM), (h) DNA + Cu(II) + curcumin (0.8 mM).


a
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OC
SC

Figure 6 : **Effect of increasing Cu(II) concentration on supercoiled plasmid DNA in the presence of curcumin.**

Reaction mixtures containing 0.75 μg plasmid pBR322 DNA, 0.1 mM curcumin and increasing concentrations of Cu(II) were incubated for 2 hours at 37°C.

Lane (a) DNA alone, (b) DNA + Cu(II) (0.8 mM), (c) DNA + curcumin, (d) DNA + curcumin + Cu(II) (0.05 mM), (e) DNA + curcumin + Cu(II) (0.1 mM), (f) DNA + curcumin + Cu(II) (0.2 mM), (g) DNA + curcumin + Cu(II) (0.4 mM), (h) DNA + curcumin + Cu(II) (0.8 mM).

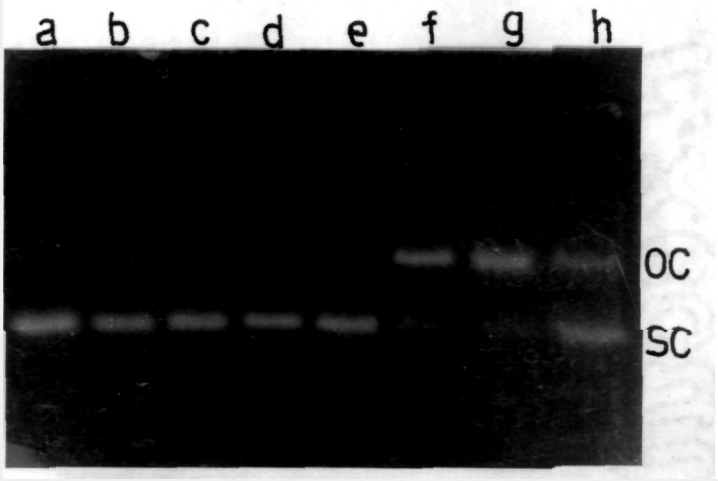
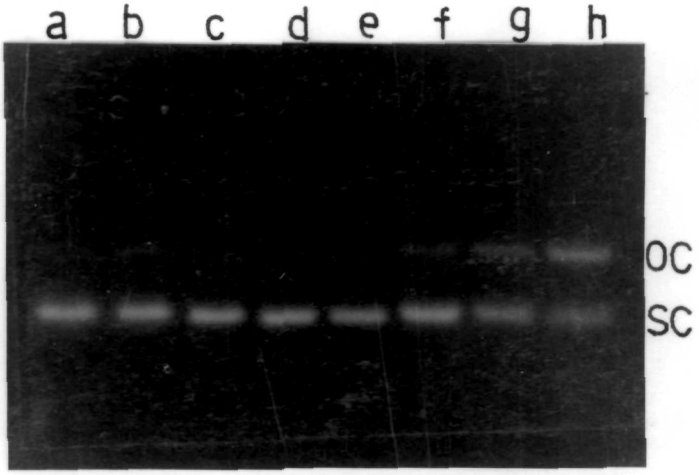

a b c d e f g h
OC
SC

Figure 7 : **Effect of increasing time of incubation on supercoiled plasmid DNA in the presence of curcumin and Cu(II)**

Reaction mixtures containing 0.75 μg plasmid pBR322 DNA, 0.1 mM curcumin and 0.2 mM Cu(II) were incubated at 37°C for different time periods.

Lane (a) DNA alone, (b) DNA + Cu(II), (c) DNA + curcumin, (d) DNA + curcumin + Cu(II), 0 min, (e) 10 min, (f) 30 min, (g) 60 min, (h) 120 min.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OC
SC

a b c d e f g h

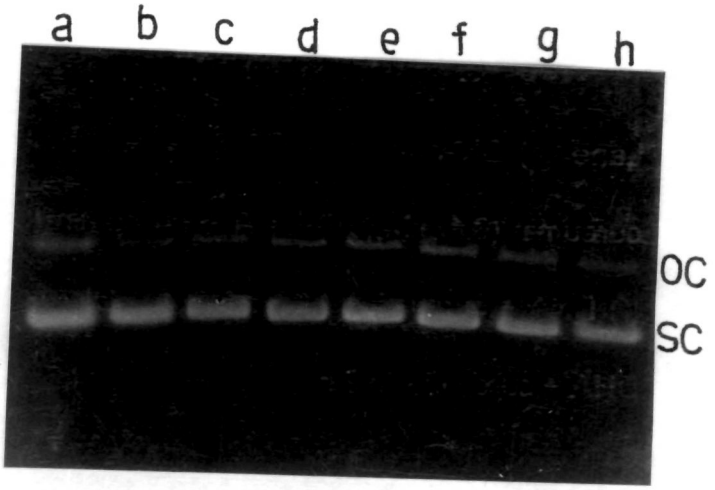
OC

SC

Figure 9 : **Effect of curcumin on supercoiled plasmid DNA in the absence of Cu(II).**

Reaction mixtures containing 1 μg plasmid pBR322 DNA and increasing concentrations of curcumin were incubated for 2 hours at room temperature. Lanes (a) - (d) correspond to reactions carried out in dark and (e) - (h) to reactions in the presence of fluorescent light.

Lane (a) DNA alone, (b) DNA + curcumin (0.05 mM), (c) DNA + curcumin (0.1 mM), (d) DNA + curcumin (0.2 mM), (e) DNA alone, (f) DNA + curcumin (0.05 mM), (g) DNA + curcumin (0.1 mM), (h) DNA + curcumin (0.2 mM).


a
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curcumin-Cu(II)-plasmid DNA reaction mixture. Neocuproine forms a stable complex with Cu(I) in aqueous solution with a stoichiometry of 2:1 (Bhat and Hadi, 1992). The inhibition of DNA breakage was examined by using a constant concentration of curcumin and Cu(II) and varying amounts of neocuproine. When increasing concentrations of neocuproine was added, a progressive decrease in the conversion of supercoiled plasmid DNA to relaxed open circles was observed (Fig. 10). In the experiment shown, a 200 μM Cu(II) concentration was used in all samples. A near complete inhibition of conversion of supercoiled DNA to relaxed form is seen at a neocuproine concentration of 400 μM, indicating that when all the Cu(I) produced is bound by neocuproine , DNA degradation does not occur.

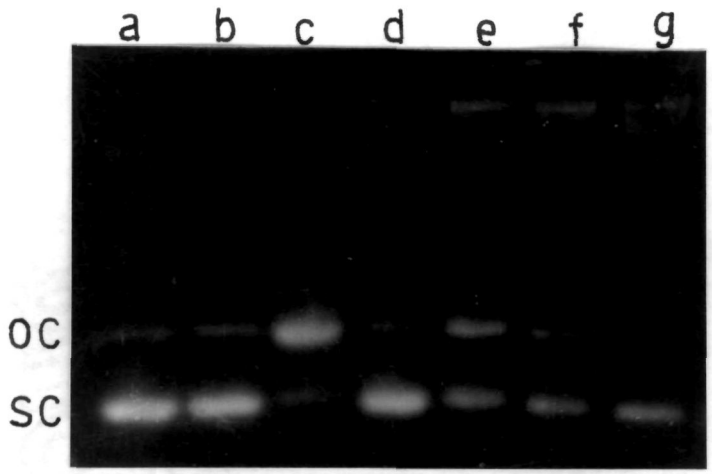
Effect of free radical scavengers on DNA breakage by curcumin and Cu(II)

Several polycyclic aromatic compounds such as flavonoids (Rahman et al., 1989), adriamycin (Eliot et al., 1984), bleomycin (Ehrenfeld et al., 1987), phenanthroline (Gutteridge and Halliwell, 1982) have been shown to cleave DNA in the presence of a metal ion and molecular oxygen. In all these reactions, active oxygen species were shown to be involved. For this reason, the effect of several free radical scavengers or quenchers on curcumin-Cu(II) mediated DNA degradation was examined. Sodium azide is a singlet oxygen ($^1O_2$) quencher; superoxide dismutase (SOD) and catalase scavenge superoxide anion (O_2^-) and

Figure 10 : Effect of increasing neocuproine concentration on curcumin-Cu(II) induced breakage of supercoiled plasmid DNA.

Reaction mixtures containing 1 μg plasmid pBR322 DNA, 0.2 mM curcumin, 0.2 mM Cu(II) and increasing concentrations of neocuproine were incubated for 3 hours at 37°C.

Lane (a) DNA alone, (b) DNA + Cu(II), (c) DNA + curcumin + Cu(II), (d) DNA + Cu(II) + neocuproine (0.6 mM), (e) DNA + curcumin + Cu(II) + neocuproine (0.1 mM), (f) 0.2 mM, (g) 0.4 mM.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OC
SC

hydrogen peroxide (H_2O_2), respectively; sodium benzoate, mannitol and potassium iodide eliminate hydroxyl free radicals (OH). As shown in figure 11, curcumin-Cu(II) induced DNA degradation was nearly completely inhibited by the addition of catalase, confirming the involvement of H_2O_2 in the reaction. Potassium iodide and sodium azide also marginally inhibited the DNA breakage reaction indicating the involvement of hydroxyl radicals and singlet oxygen, respectively.

Photogeneration of superoxide anion by curcumin

Figure 12 shows the generation of superoxide anion by curcumin in visible light. An increase in the absorbance at 560 nm is observed on reduction of nitroblue tetrazolium (NBT) to a formazan by superoxide. The reaction is almost completely inhibited in presence of superoxide dismutase (SOD) indicating that the method genuinely assays the superoxide free radical.

Production of hydroxyl radicals by curcumin and Cu(II)

In this experiment, it is seen that hydroxyl radical is generated by curcumin. Table II shows the effect of increasing curcumin concentration on the generation of hydroxyl radicals as determined by the formation of hydroxylated salicylic acid. The formation of hydroxyl radicals increases with an increase in the concentration of curcumin.

Formation of hydrogen peroxide by curcumin

Figure 11 : Effect of free radical scavengers on curcumin-Cu(II) induced breakage of supercoiled plasmid DNA.

Reaction mixtures containing 1 μg plasmid pBR322 DNA, 0.2 mM curcumin, 0.2 mM Cu(II) and quenchers were incubated for 3 hours at 37°C.

Lane (a) DNA alone, (b) DNA + curcumin + Cu(II), (c) DNA + curcumin + Cu(II) + KI (50 mM), (d) DNA + curcumin + Cu(II) + NaN_3 (50 mM), (e) DNA + curcumin + Cu(II) + benzoate (50 mM), (f) DNA + curcumin + Cu(II) + mannitol (50 mM), (g) DNA + curcumin + Cu(II) + SOD (0.1 mg/ml), (h) DNA + curcumin + Cu(II) + catalase (0.1 mg/ml).

a b c d e f g h

OC

SC

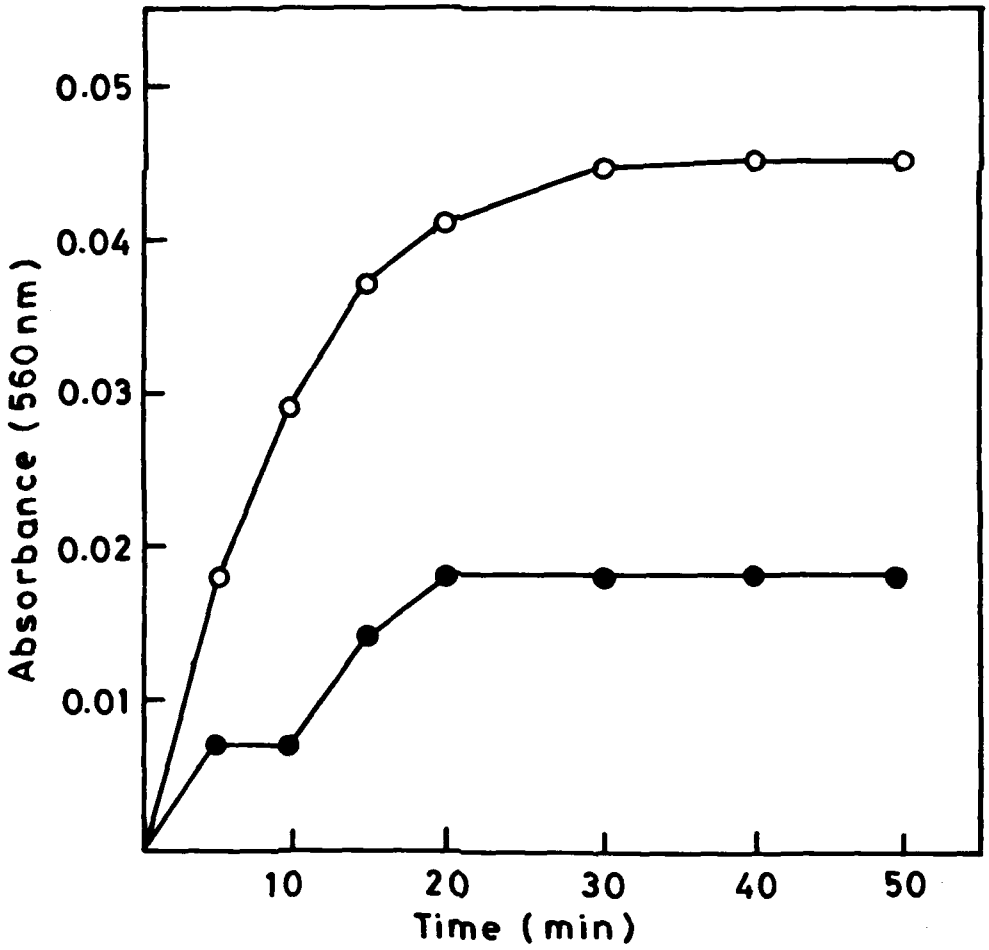

0.05
0.04
0.03
0.02
0.01
Absorbance (560nm)
10
20
30
40
50
Time (min)

TABLE II

Formation of hydroxyl radicals as a function of curcumin concentration.

Curcumin (μM)	Hydroxylated product (n mol)
10	82.5
20	116.0
30	132.9
40	175.1
50	208.6

Values of curcumin shown are final reaction concentrations. Cu(II) was used at a final concentration of 0.1 mM. The incubation was done for 2 hours at room temperature in fluorescent light. Reaction conditions are described in Methods.

The pathway for the generation of hydroxyl radicals involves hydrogen peroxide as intermediate. Hydrogen peroxide in turn gives rise to hydroxyl radical either by the Haber-Weiss or Fenton reactions :

i) $O_2^{\cdot} + 2H^{+} \rightarrow H_2O_2 + O_2$

$O_2^{\cdot} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^{-}$ (Haber-Weiss Reaction)

ii) $H_2O_2 + Cu(I) \rightarrow OH^{\cdot} + OH^{-} + Cu(II)$ (Fenton Reaction)

In this experiment, I have determined the H_2O_2 production capacity of curcumin. The method used involves the oxidation of titanium to pertitanic acid by hydrogen peroxide (Nakayama et al., 1983). Figure 13 shows the production of H_2O_2 with increasing concentrations of curcumin. It is seen that hydrogen peroxide formation increases with the concentration of curcumin. In the presence of catalase, H_2O_2 is not produced confirming that the method employed measures hydrogen peroxide in this assay.

Curcumin-DNA interaction

The absorbance of curcumin with a maximum at 420 nm is affected by the addition of increasing DNA concentrations (Fig. 14), showing both enhancement (hyperchromic shift) and quenching (hypochromic shift) in absorption depending on the concentration,and also a shift in the absorption maximum (bathochromic shift) to about 440 nm. These results indicate that curcumin is capable of binding to double stranded DNA.

Figure 13 : Generation of hydrogen peroxide as a function of increasing curcumin concentration.

Incubation was done for 1 hour at room temperature. Reaction conditions are described in Methods.

(o) curcumin alone

(●) curcumin + catalase

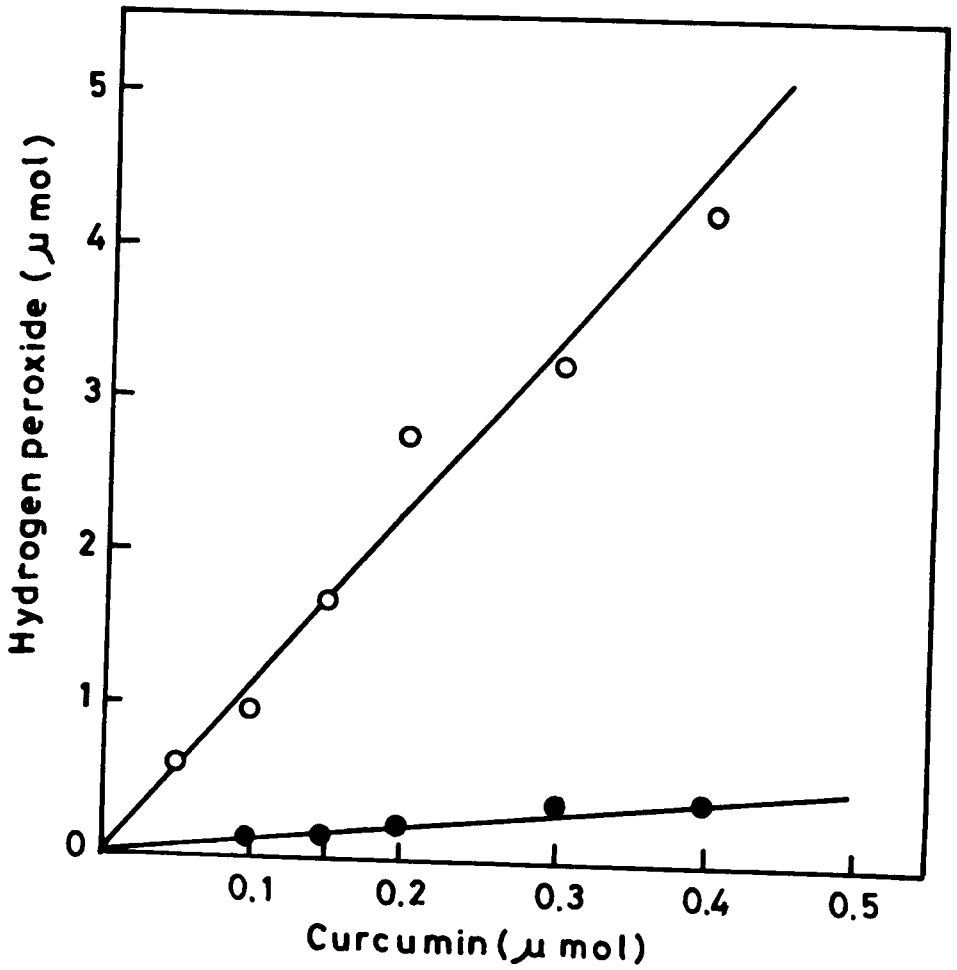
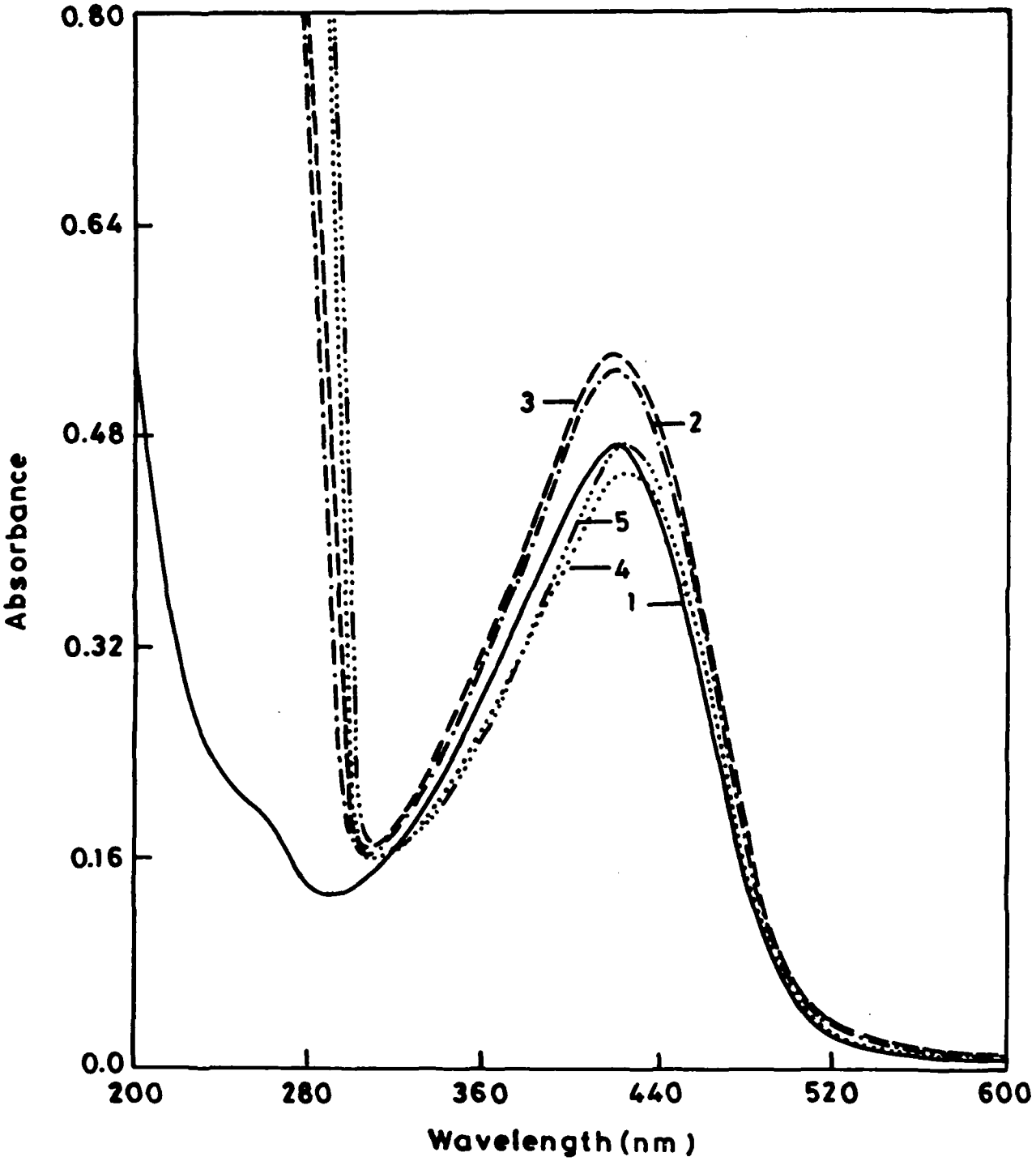

Hydrogen peroxide (μ mol)
0
1
2
3
4
5
0.1
0.2
0.3
0.4
0.5
Curcumin (μ mol)

Figure 14 : **Effect of increasing concentration of calf thymus DNA on the absorption spectrum of curcumin.**

The concentration of curcumin in the reaction mixture was 25 μM in 10 mM Tris-HCl buffer (pH 8.0). The ratio of curcumin to DNA base pairs is:

Trace 1 (—) curcumin alone; 2 (—·—) 1:5; 3 (- -) 1:10; 4 (····) 1:20; 5 (···—) 1:40


0.80
0.64
0.48
0.32
0.16
0.0
Absorbance
200
280
360
440
520
600
Wavelength (nm)
1
2
3
4
5

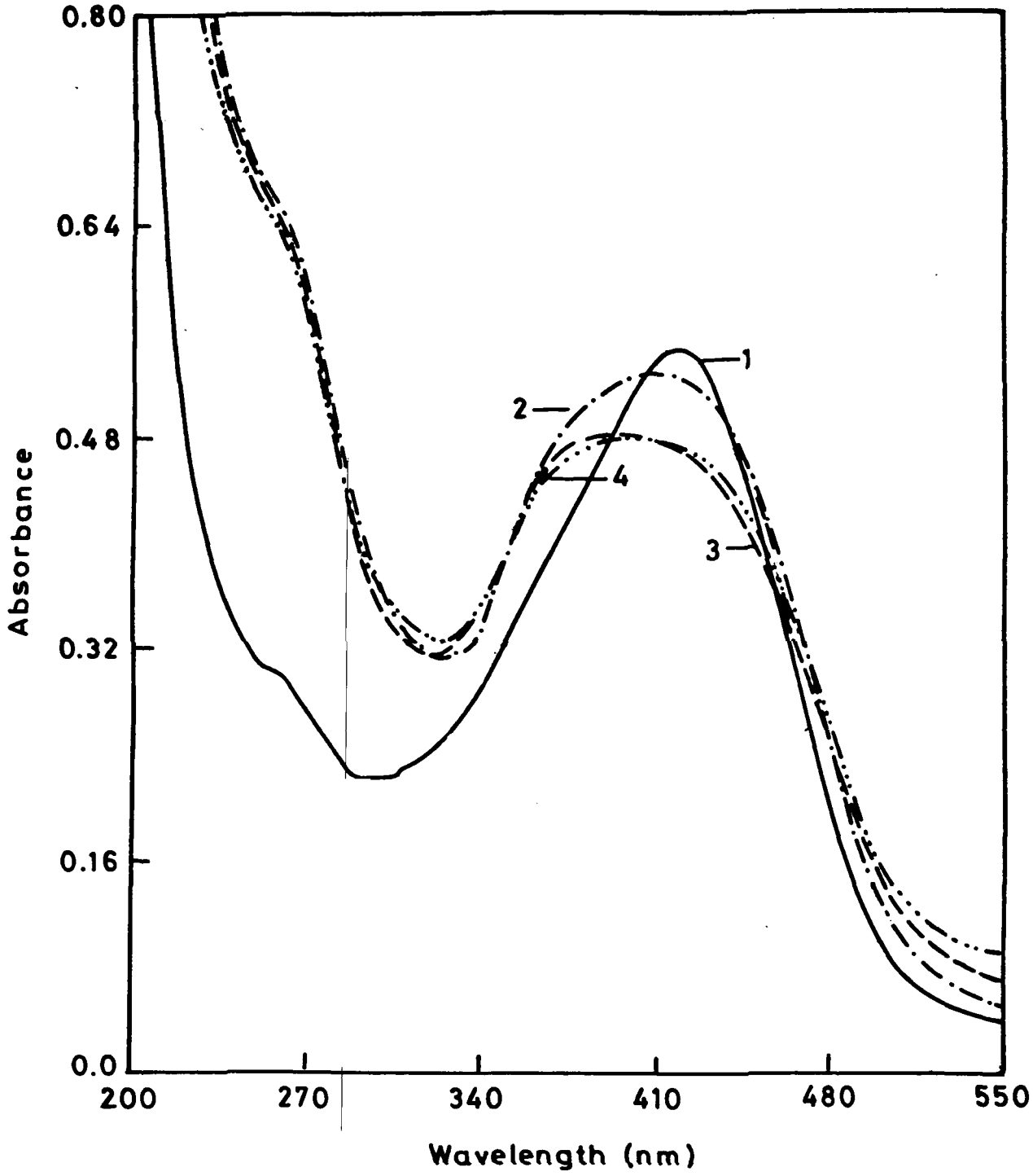
Curcumin-Cu(II) interaction

When curcumin and Cu(II) solutions were mixed, a shift occured in the λmax of curcumin from 420 nm to 390 nm (hypsochromic shift). The broad peak at 390 nm also decays with time as seen in figure 15. These results are indicative of the binding of Cu(II) to curcumin. Taken together with the previous results it would appear that the formation of a ternary complex is possible between DNA, curcumin and Cu(II).

Figure 15 : **Time course of absorption spectral change in curcumin induced by the addition of Cu(II).**

The concentration of curcumin and Cu(II) were 25 μM and 100 μM respectively, in 10 mM Tris-HCl buffer (pH 8.0). Absorption spectra were recorded at different time periods after the addition of Cu(II).

Trace 1 (——) curcumin alone; 2 (–·–) 1 min; 3 (– –) 10 min; 4 (··· –) 30 min.


0.80
0.64
0.48
0.32
0.16
0.0
Absorbance
200
270
340
410
480
550
Wavelength (nm)
1
2
3
4

DISCUSSION
(PART I)

DISCUSSION (Part I)

The results presented here lead to the following conclusions -

i) Curcumin in the presence of Cu(II) and molecular oxygen causes degradation of calf thymus DNA, ii) Curcumin generates single stranded breaks in plasmid DNA in the presence of transition metal ions, mainly Cu(II) and to some extent Fe(II), iii) Cu(II) is reduced to Cu(I), the latter being an essential intermediate in the DNA cleavage reaction, iv) The proximal DNA cleaving agents are the active oxygen species: hydroxyl radicals, hydrogen peroxide and singlet oxygen appear to be involved.

The degradation of DNA by a number of DNA - binding drugs such as bleomycin (Ehrenfeld et al., 1987), rifamycin (Quinlan and Gutteridge, 1987), adriamycin (Eliot et al., 1984), the flavonoid quercetin (Rahman et al., 1989) is dependent upon metal ions and is considered to rely on the generation of oxygen derived free radicals (ROS). It appears that curcumin-Cu(II) induced DNA breakage is similar in nature and does not require a reducing agent. It is proposed that a ternary complex of the drug curcumin, DNA and Cu(II) is formed, in which reduction of Cu(II) to Cu(I) occurs. The conversion of supercoiled molecule to the relaxed form is the result of a single nick in the DNA molecule. A second nick close to the first would give rise to the linear form. However, in the present case, no linear forms of plasmid DNA were observed.

There are two alternative routes for the generation of hydroxyl radicals :

i) $O_2^- + H_2O_2 \rightarrow OH^\cdot + OH^- + O_2$

ii) $H_2O_2 + Cu(I) \rightarrow OH^\cdot + OH^- + Cu(II)$

Reaction (i) is generally referred to as the Haber-Weiss reaction (modified) and (ii) as the Fenton reaction. It is known that the generation of superoxide anion may lead to the formation of hydrogen peroxide. Alternatively, the superoxide anion undergoes dismutation to form H_2O_2 and O_2 in aqueous solutions (Halliwell and Gutteridge, 1984) :

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The formation of singlet oxygen may occur through the Haber-Weiss reaction or the interaction of superoxide anion with hydroxyl radicals (Badway and Karnovsky, 1980).

$$O_2^- + H_2O_2 \xrightarrow{Metal} {}^1O_2 + OH^\cdot + OH^-$$

$$O_2^- + OH^\cdot \longrightarrow {}^1O_2 + OH^-$$

Curcumin is capable of reducing Cu(II) to Cu(I), which is an intermediate in the DNA-degradation reaction. The drug catalysed reduction of transition metals has been implicated in DNA damage reactions by several other naturally occurring compounds (Naseem et al., 1993; Bhat and Hadi, 1992; Zaidi and Hadi, 1992; Rahman et al., 1989).

Ascorbic acid, flavonoids and curcumin are generally considered to be dietary antioxidants (Namiki, 1990; Perchellet and Perchellet, 1989). Curcumin, a polyphenol and a naturally occurring phytochemical, is a major chemical

constituent of the ground and dried rhizome of Curcuma longa commonly called turmeric. Curcumin stimulates quercetin induced nuclear DNA damage, protein degradation and lipid peroxidation in the presence of copper or iron and therefore acts as a pro-oxidant (Sahu and Washington, 1992). It seems that it probably undergoes autoxidation in the presence of oxygen and transition metal ions. This autoxidation generates ROS, and hence its pro-oxidant behaviour. Its molecular structure would allow it to react with oxygen free radicals, produced by other compounds, providing it with radical-trapping properties. Thus, curcumin has the potential of acting both as pro- and antioxidant, depending upon the redox state of the biological environment. In the cellular environment, these two opposing effects may be competitive; therefore, it may have a dual role in mutagenesis and carcinogenesis (Kahl, 1986).

RESULTS
(PART II)

RESULTS (Part II)

Cleavage of pBR322 DNA by Curcuminoids and Cu(II)

It was previously shown that curcumin causes degradation of supercoiled plasmid DNA (Fig. 4). In figure 16, the rate of hydrolysis of supercoiled plasmid DNA by purified curcuminoids (curcumin demethoxy and bisdemethoxycurcumin) in the presence of Cu(II) has been compared. Figure 16A shows the EtBr strained pattern of pBR322 DNA treated with increasing concentrations of curcumin in presence of 100 μM Cu(II). As expected, curcumin converted supercoiled plasmid DNA to relaxed open circles and there was a near complete conversion at 200 μM curcumin concentration. However, it may be noted that as in fig. 5, there is inhibition of strand breakage activity at higher concentrations of curcumin.

Demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC) had a rather limited effect on supercoiled pBR322 DNA. Figure 16B shows the conversion of supercoiled plasmid DNA to open circular form in presence of 100 μM Cu(II) and increasing concentrations of dmC. It is seen that there is a similar banding pattern observed for concentrations ranging from 0.1 to 0.3 mM dmC. But at 500 μM dmC concentration, there is greater conversion of supercoiled DNA to open circular molecules. Similarly, with increasing concentrations of

Figure 16 : Effect of increasing curcumin, demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC) concentration on supercoiled plasmid DNA in the presence of Cu(II).

Reaction mixtures containing 0.5 μg plasmid pBR322 DNA, 0.1 mM Cu(II) and increasing concentration of curcuminoids were incubated for 2 hours at room temperature (OC - open circular DNA, SC - supercoiled DNA).

A : Lane (a) DNA alone, (b) DNA + curcumin (0.5 mM), (c) DNA + Cu(II) + curcumin (0.1 mM), (d) DNA + Cu(II) + curcumin (0.2 mM), (e) DNA + Cu(II) + curcumin (0.3 mM), (f) DNA + Cu(II) + curcumin (0.5 mM)

B : Lane (a) DNA alone, (b) DNA + dmC (0.5 mM), (c) DNA + Cu(II) + dmC (0.1 mM), (d) DNA + Cu(II) + dmC (0.2 mM), (e) DNA + Cu(II) + dmC (0.3 mM), (f) DNA + Cu(II) + dmC (0.5 mM).

C : Lane (a) DNA alone, (b) DNA + bdmC (0.5 mM), (c) DNA + Cu(II) + bdmC (0.1 mM), (d) DNA + Cu(II) + bdmC (0.2 mM), (e) DNA + Cu(II) + bdmC (0.3 mM), (f) DNA + Cu(II) + bdmC (0.5 mM).

bdmC, there is a dose dependent conversion to open circular form (Fig. 16C) and at a higher concentration (0.5 mM) the reaction is inhibited.

Figure 17 gives the comparative effect of curcuminoids on supercoiled plasmid DNA in a single experiment. It is seen that in the case of curcumin a major proportion of supercoiled form was converted to open circular molecules (lane e). However, there was partial conversion to open circular form in case of dmC (lane f) but only a limited effect was seen with bdmC (lane g).

Curcumin, dmC and bdmC alone in the absence of Cu(II) do not lead to any DNA cleavage even after a prolonged incubation period of 6 hours. The result with curcumin as a function of concentration is shown in figure 18.

Production of $OH^{\cdot}$ radicals by curcuminoids

Similar to curcumin (Table II), the other curcuminoids namely dmC and bdmC also generate $OH^{\cdot}$ both in the absence and presence of Cu(II) (Table III). The production is considerably enhanced in the presence of Cu(II) in all the three cases. Figure 19 compares the rate of formation of $OH^{\cdot}$ with increasing concentration of curcuminoids. As seen, the rate observed is in the order curcumin > dmC > bdmC. The generation of $OH^{\cdot}$ is inhibited by benzoate which confirms the validity of the method to measure hydroxyl radical formation.

Generation of H_2O_2 by curcuminoids

As described previously, the pathway for the generation of $OH^{\cdot}$ involves H_2O_2 as intermediate. The superoxide anion and H_2O_2 interact in the presence of

Figure 17 : Effect of curcumin, demethoxycurcumin (dmC) and bisdemethoxy-curcumin (bdmC) on supercoiled plasmid DNA in the presence of Cu(II).

Reaction mixtures containing 0.5 μg plasmid pBR322 DNA, 0.1 mM Cu(II) and 0.1 mM curcuminoids were incubated for 3 hours at room temperature.

Lane (a) DNA alone, (b) DNA + curcumin, (c) DNA + dmC, (d) DNA + bdmC, (e) DNA + Cu(II) + curcumin, (f) DNA + Cu(II) + dmC, (g) DNA + Cu(II) + bdmC.

a b c d e f g

OC

SC

a b c d e f

OC
SC

TABLE III

Effect of Cu(II) on the generation of hydroxyl radicals by curcumin, demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC)

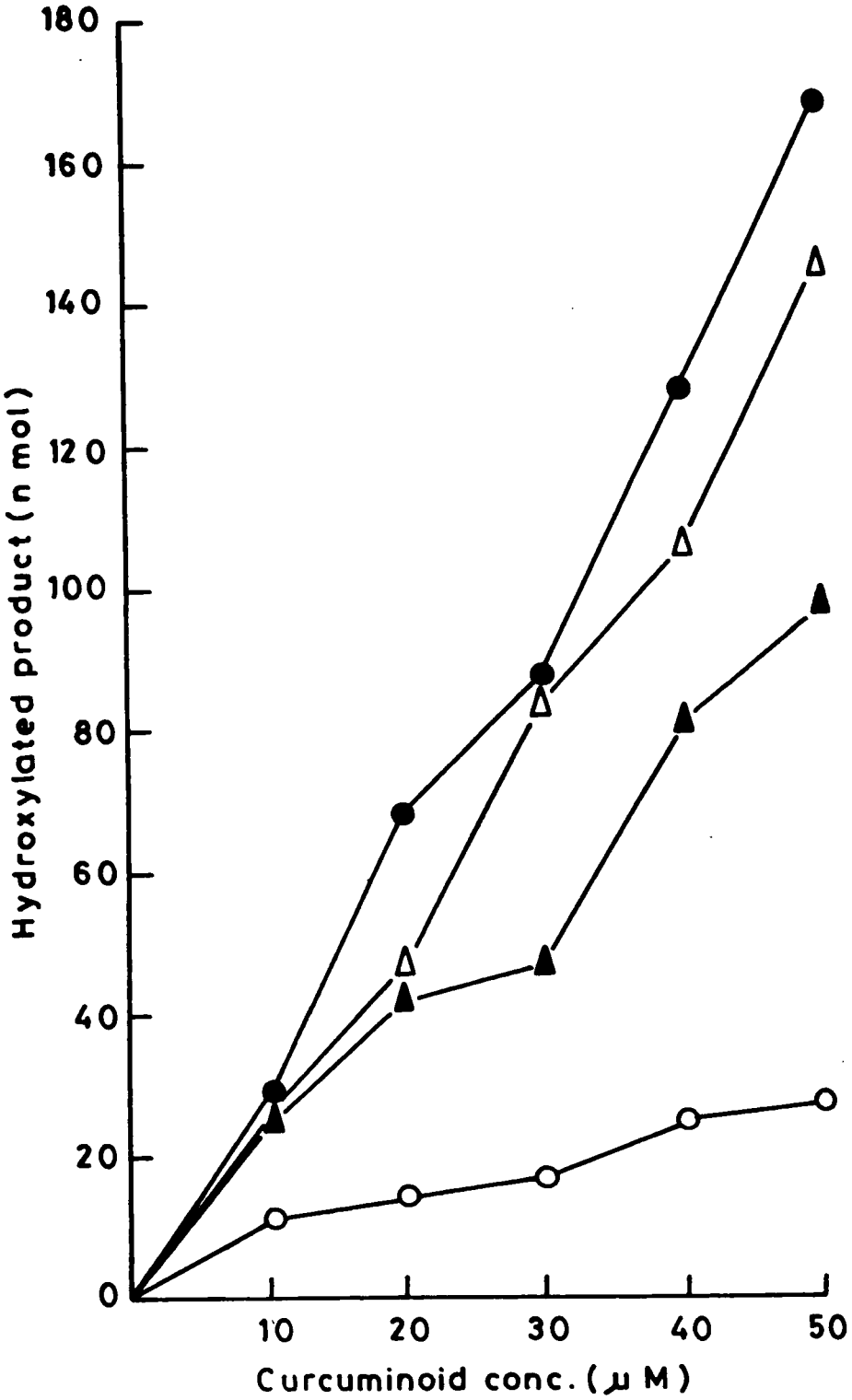
Curcuminoids	Hydroxylated product (n mol)	
	Absence of Cu(II)	Presence of Cu(II)
Curcumin	92.6	168.6
dmC	57.5	128.6
bdmC	13.2	29.2

The final concentration of curcuminoids and Cu(II) in the reaction mixture was 50 μM and 100 μM, respectively.

Figure 19 : Production of hydroxyl radicals as a function of increasing curcumin, demethoxycurcumin (dmC) and bisdemethoxy-curcumin (bdmC) concentration.

The reaction mixtures containing increasing concentration of curcuminoids (10-50 μM) were incubated for 2 hours at room temperature in the presence of light. Reaction conditions are described in Methods.

(●) curcumin, (Δ) dmC, (▲) bdmC, (o) curcumin + sodium benzoate (5 mM).


180
160
140
120
100
80
60
40
20
0
Hydroxylated product (n mol)
10
20
30
40
50
Curcuminoid conc. (μ M)

copper salts to generate OH· in the Haber-Weiss reaction (Beuchamp and Fridovich, 1970).

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$O_2^- + H_2O_2 \xrightarrow{Cu(II)} O_2 + OH\cdot + OH^-$$

Therefore, it was of interest to compare the rate of production of H_2O_2 by different curcuminoids. The method used, as mentioned, relies on the colourimetric assay of a Ti(IV) peroxysulphate. As seen in figure 20, all the curcuminoids are capable of generating H_2O_2 and the relative rate of formation is qualitatively similar to that of OH· formation. The reaction is also dose dependent and is significantly inhibited in the presence of catalase confirming that the procedure genuinely measures H_2O_2.

Changes in the absorption of curcuminoids upon reaction with Cu(II)

The curcuminoids used in this study exhibit a visible absorption spectrum with a major peak around 415 nm. In the case of curcumin (Fig. 21A), the addition of Cu(II) results in an instantaneous quenching of the major band which is indicative of the formation of a complex with copper. This is similar to the changes seen with the unpurified curcumin preparation which contains all three curcuminoids (Fig. 15). The absorption spectrum changes further as a function of time with gradual quenching of the peak. The quenched peaks also exhibit a slight hypsochromic shift. These results further indicate that the formation of the

Figure 20 : Generation of hydrogen peroxide as a function of increasing curcumin, demethoxycurcumin (dmC) and bisdemethoxy-curcumin (bdmC) concentration.

The reaction mixtures containing increasing concentration of curcuminoids (0.1 - 0.3 μmol) were incubated for 2 hours at room temperature. Reaction conditions are described in Methods.

(●) curcumin, (Δ) dmC, (▲) bdmC, (o) curcumin + catalase (0.1 mg/ml).

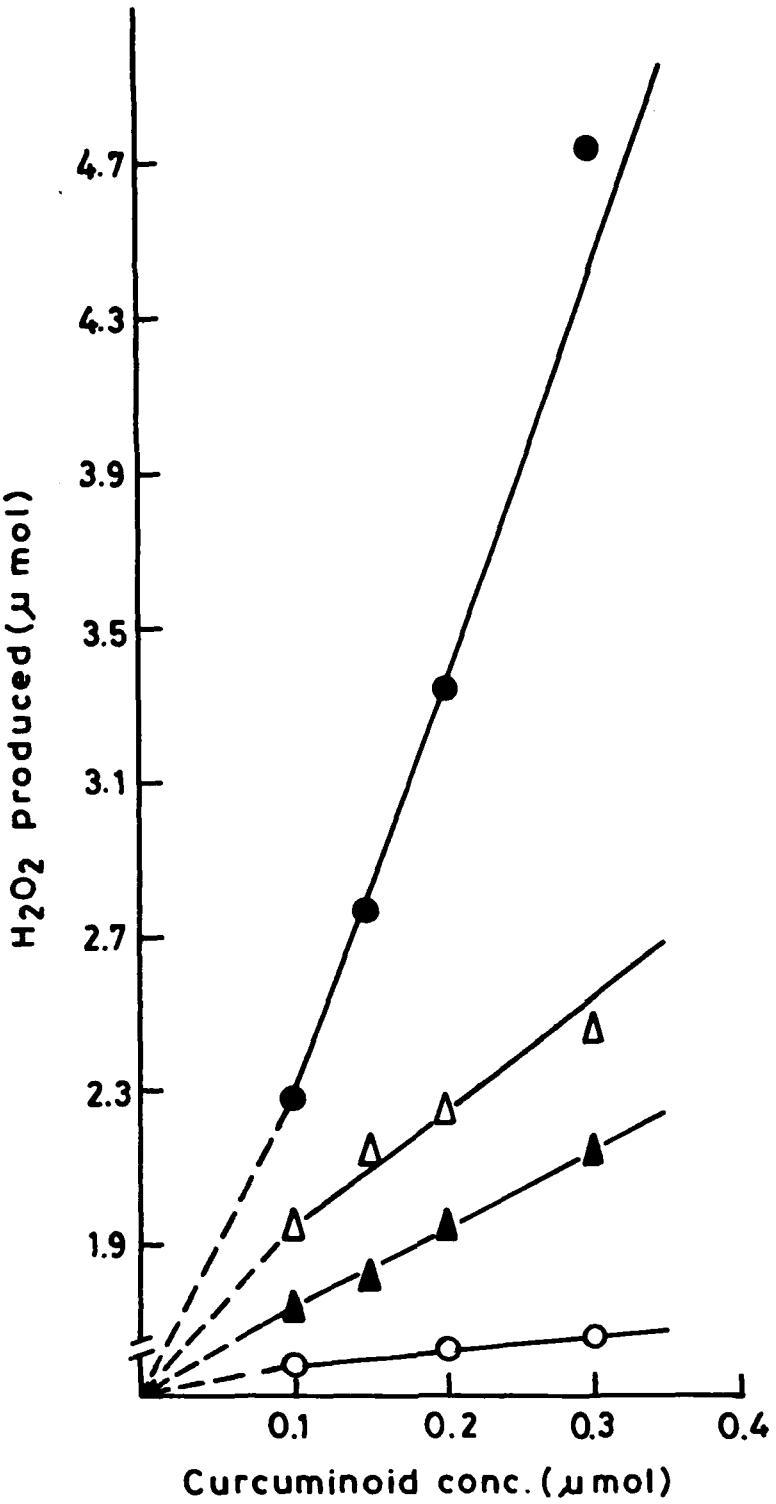
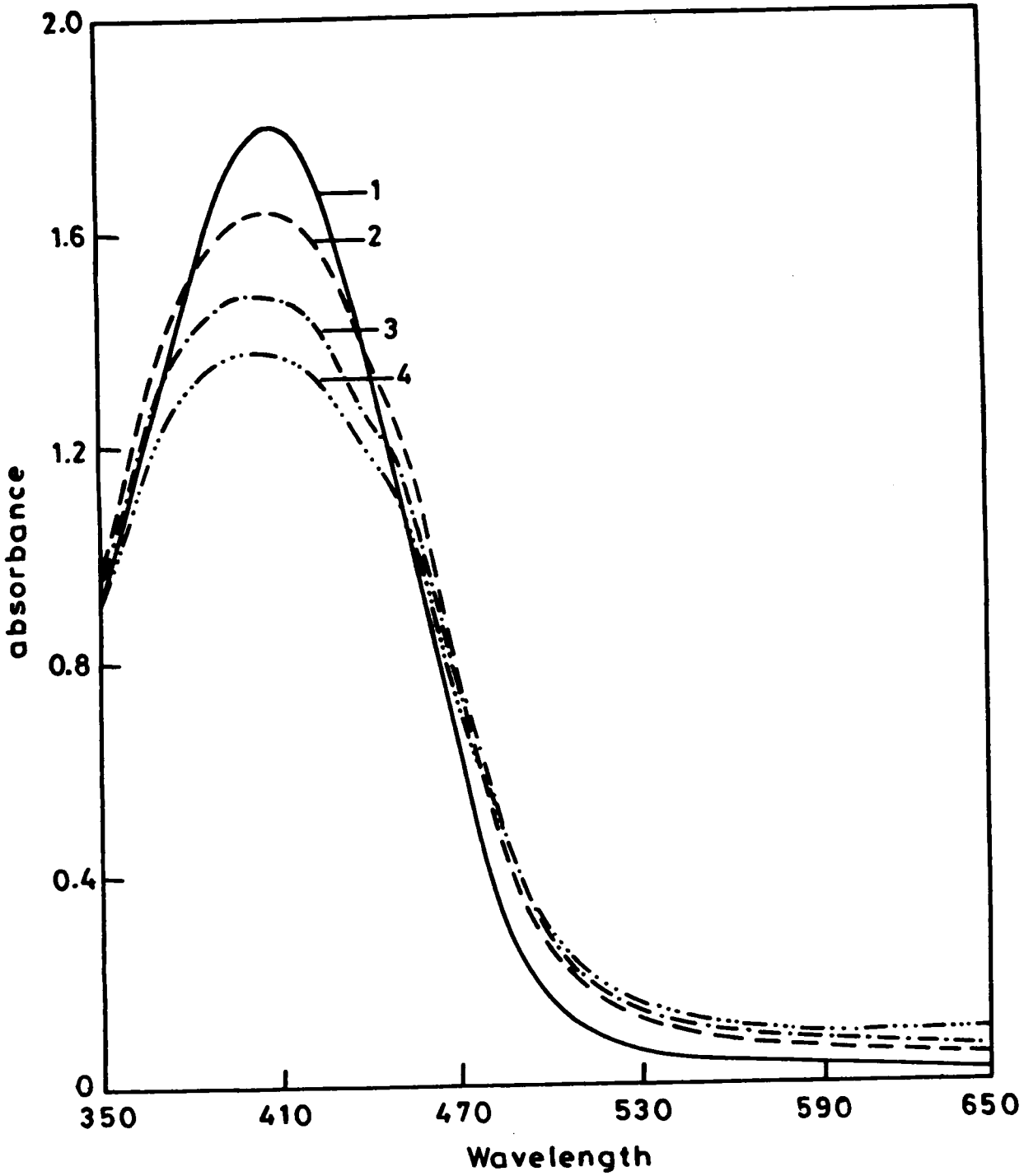

4.7
4.3
3.9
3.5
3.1
2.7
2.3
1.9
H_2O_2 produced (μ mol)
0.1
0.2
0.3
0.4
Curcuminoid conc. (μ mol)

Figure 21A : Time course of absorption spectral change in curcumin induced by the addition of Cu(II).

Spectra of a solution containing curcumin (50 μM) and Cu(II) (100 μM) in 10 mM Tris-HCl buffer (pH 7.5), were recorded at different time periods after the addition of Cu(II). 1 (——) curcumin alone, 2 (--) 0 min, 3 (–·–) 10 min, 4 (···–) 30 min.


2.0
1.6
1.2
0.8
0.4
0
absorbance
1
2
3
4
350
410
470
530
590
650
Wavelength

complex is a slow reaction and is not fully complete even after 30 minutes. Figures 21B and C depict similar experiments with dmC and bdmC and essentially show similar results. It is however seen that the extent of quenching varies in the case of dmC and bdmC.

Absorption spectra of curcuminoid-DNA complex

Figure 22A shows the visible absorption spectrum of curcumin from 350-650 nm, in the presence of increasing concentrations of DNA. An enhancement in the absorption of curcumin is initially observed which is quenched at a higher concentration of DNA. Figures 22B and C show similar experiments with dmC and bdmC, respectively. However, the enhancement in the absorption of dmC is observed with all DNA concentrations used. In contrast to curcumin and dmC, only quenching is seen in the case of bdmC with all concentrations of DNA.

Antioxidant Effect Of Curcuminoids

There are over 100 diseases in which free radicals are thought to be involved including acute respiratory distress syndrome (ARDS), rheumatoid arthritis, atherosclerosis, myocardial infarction, hemorrhagic shock, AIDS, etc (Knight, 1995; Martinez-Cayuela, 1995; Halliwell and Cross, 1991; Reilly et al., 1991). Important free radicals in living organisms include hydroxyl ($OH^{\cdot}$) and superoxide radicals ($O_2^{\cdot-}$). The term 'reactive oxygen species' (ROS) is often used in the biomedical literature to include not only oxygen centered radicals such as $OH^{\cdot}$ and $O_2^{\cdot-}$ but also non-radical derivatives of oxygen including

Figure 21B: Time course of absorption spectral change in demethoxycurcumin (dmC) induced by the addition of Cu(II).

Spectra of a solution containing dmC (50 μM) and Cu(II) (100 μM) in 10 mM Tris-HCl buffer (pH 7.5), were recorded at different time periods after the addition of Cu(II). 1 (—) dmC alone, 2 (– –) 0 min, 3 (–·–) 10 min, 4 (···—) 30 min.

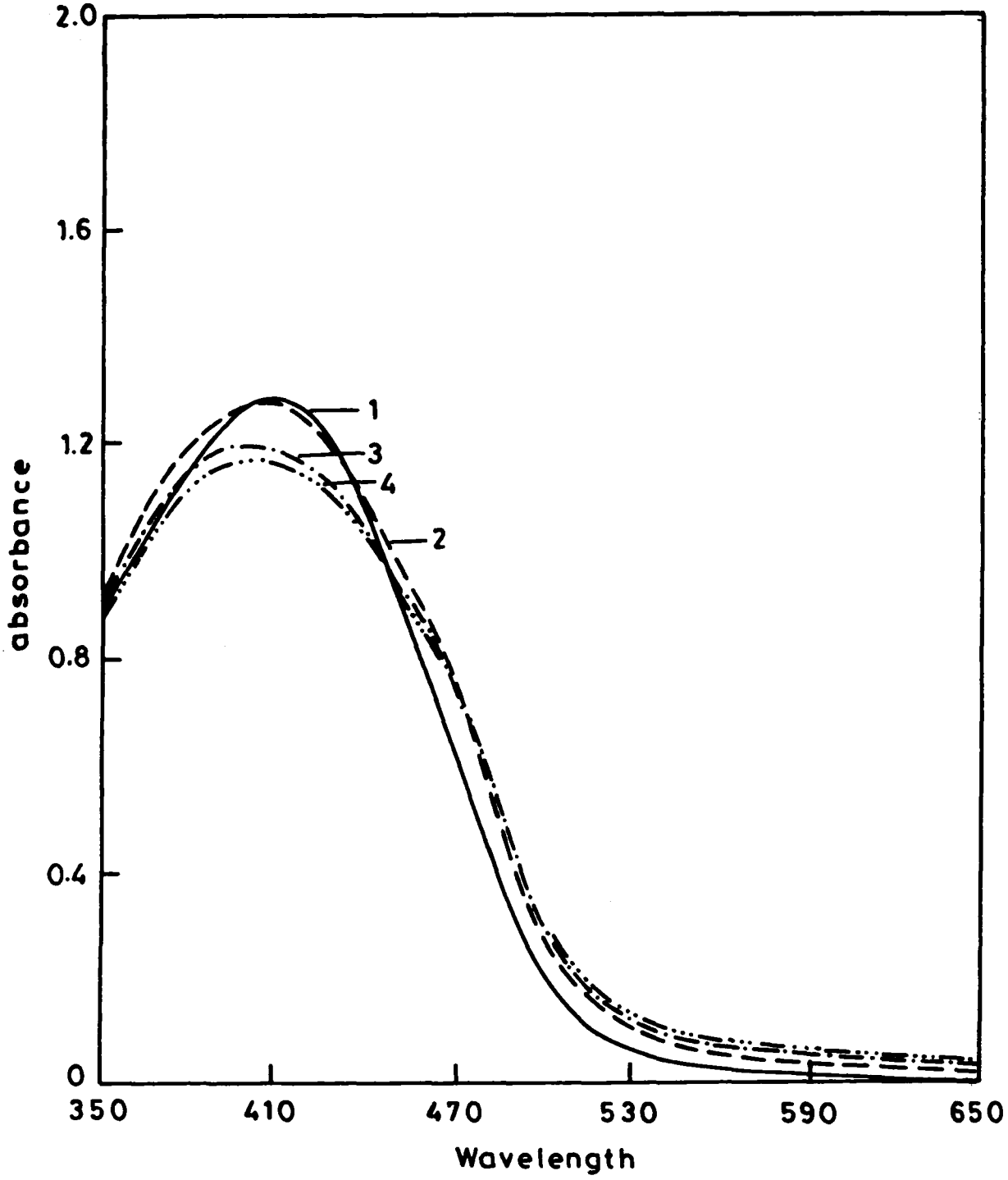

2.0
1.6
1.2
0.8
0.4
0
absorbance
1
2
3
4
350
410
470
530
590
650
Wavelength

Figure 21C : Time course of absorption spectral change in bisdemethoxycurcumin (bdmC) induced by the addition of Cu(II).

Spectra of a solution containing bdmC (50 μM) and Cu(II) (100 μM) in 10 mM Tris-HCl buffer (pH 7.5), were recorded at different time periods after the addition of Cu(II). 1 (——) bdmC alone, 2 (— –) 0 min, 3 (– • –) 10 min, 4 (··· —) 30 min.

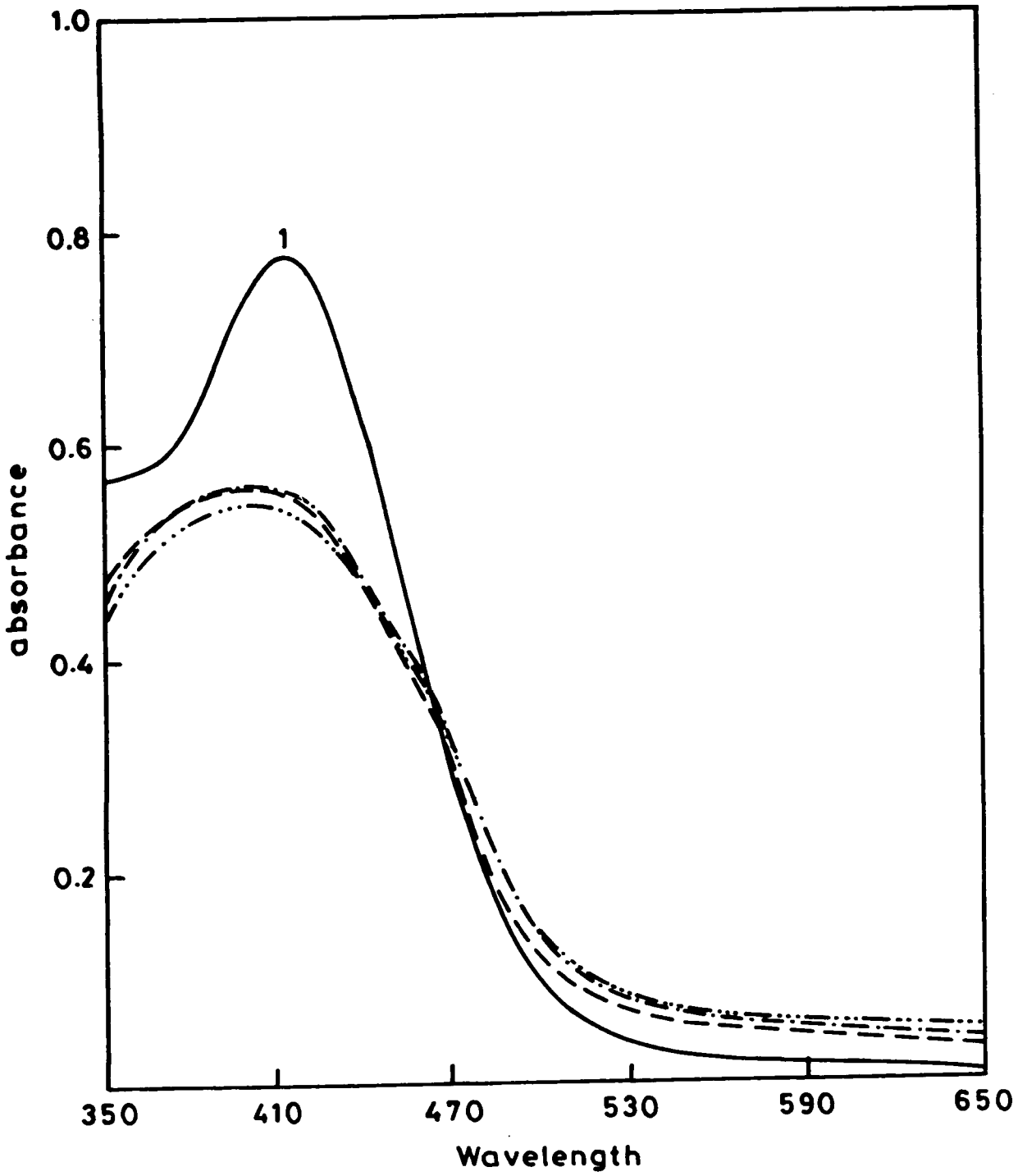

1.0
0.8
0.6
0.4
0.2
absorbance
1
350
410
470
530
590
650
Wavelength

Figure 22A : Effect of increasing concentration of calf thymus DNA on the absorption spectrum of curcumin.

The concentration of curcumin in the reaction mixture was 25 μM in 10 mM Tris-HCl buffer, pH 7.5. The ratio of curcumin to DNA base-pairs is:

1 (—) curcumin alone; 2 (– –) 1:1; 3 (– · –) 1:5; 4 (· · —) 1:10; 5 (· · · –) 1:20.

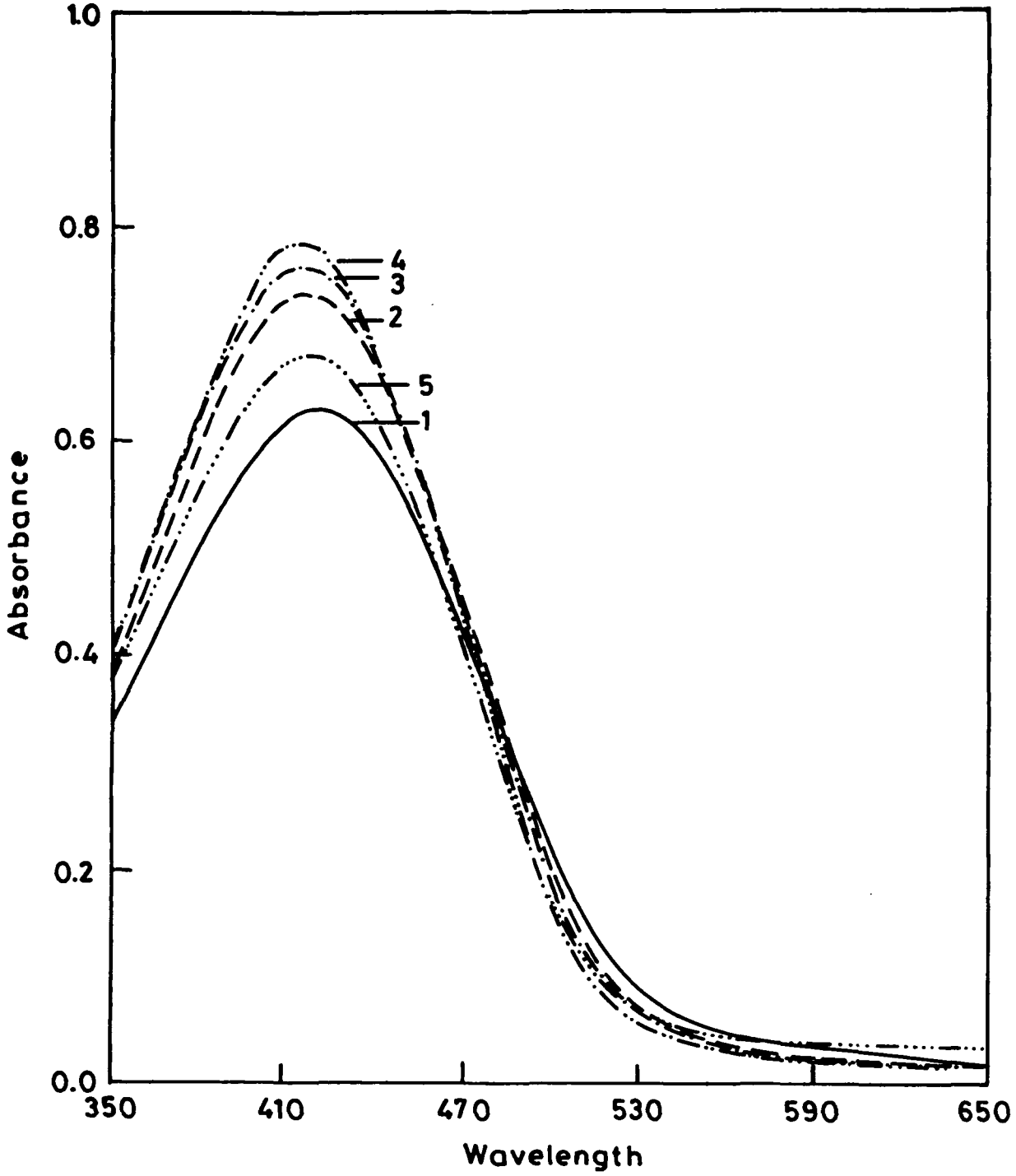

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
4
3
2
5
1
350
410
470
530
590
650
Wavelength

Figure 22B : Effect of increasing concentration of calf thymus DNA on the absorption spectrum of demethoxycurcumin (dmC).

The concentration of dmC in the reaction mixture was 25 μM in 10 mM Tris-HCl buffer, pH 7.5. The ratio of dmC to DNA base-pairs is:

1 (—) dmC alone; 2 (— —) 1:1; 3 (— · —) 1:5; 4 (·· —) 1:10; 5 (··· —) 1:20.

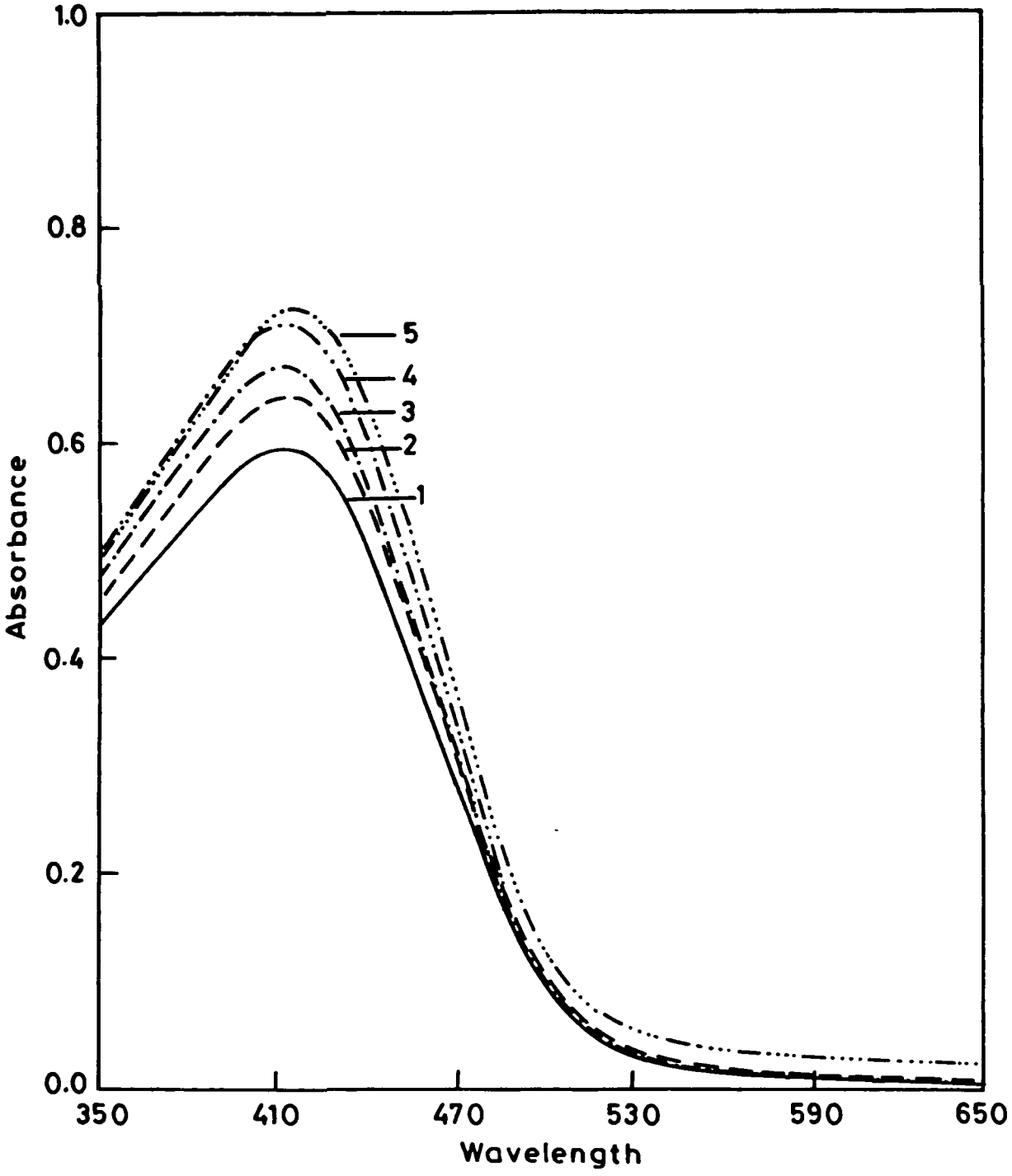
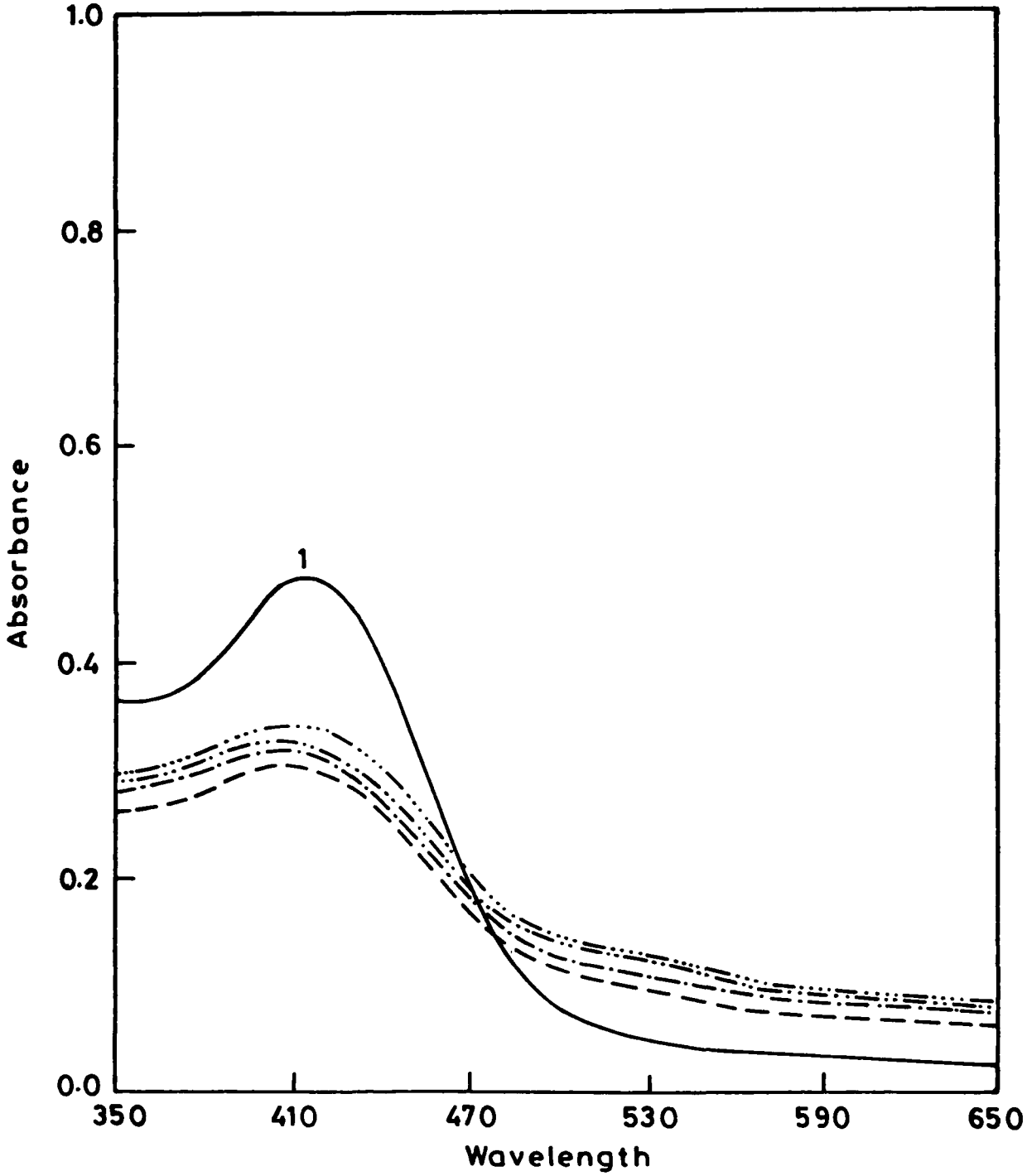

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
5
4
3
2
1
350
410
470
530
590
650
Wavelength

Figure 22C : Effect of increasing concentration of calf thymus DNA on the absorption spectrum of bisdemethoxycurcumin (bdmC).

The concentration of bdmC in the reaction mixture was 25 μM in 10 mM Tris-HCl buffer, pH 7.5. The ratio of bdmC to DNA base-pairs is:

1 (—) bdmC alone; 2 (– –) 1:1; 3 (– • –) 1:5; 4 (•• —) 1:10; 5 (••• –) 1:20.


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
1
350
410
470
530
590
650
Wavelength

hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3), because they can easily lead to free radical reactions (Halliwell et al., 1992; Aruoma and Halliwell, 1991). Humans have defences against ROS induced damage which include the enzymes catalase and glutathione peroxidase, both of which remove H_2O_2, and superoxide dismutase (SOD) which catalyses the dismutation of $O_2^{\cdot}$ to give H_2O_2 and O_2. In addition, some low molecular mass agents such as uric acid, β-carotene, vitamin C, vitamin E, glutathione may also act as antioxidants in the human body (Halliwell, 1994; Yu, 1994; Halliwell et al., 1992).

In the following experiments, the relative capacity of different curcuminoids to inhibit DNA cleavage by singlet oxygen and the hydroxyl radical have been compared. As a source of $^1O_2$ generation the photosensitization of riboflavin was used (Joshi, 1985) and the OH was produced by the Fe(II)-EDTA-H_2O_2 (Prigodich and Martin, 1990; Tullius and Dombroski, 1986) and kojic acid-Fe(II) systems (Bhat and Hadi, 1992).

Production of singlet oxygen by riboflavin and its inhibition by curcuminoids

The ability of riboflavin to form $^1O_2$ was determined by monitoring the bleaching of p-nitrosodimethylaniline (pRNO) according to Joshi (1985). Evidence for the formation of $^1O_2$ was obtained by examining the progress of the reaction in presence of sodium azide, a specific quencher for $^1O_2$. A near

complete inhibition of $^1O_2$ production was observed. Curcuminoids (at a concentration of 0.1 mM) were also found to inhibit the production of $^1O_2$ to various extents. As seen in figure 23, curcumin was found to be the most effective quencher, while bdmC was least inhibitive.

Inhibition of singlet oxygen induced cleavage of supercoiled plasmid DNA by curcuminoids

Singlet oxygen generated by photoilluminated riboflavin cleaves supercoiled plasmid DNA as seen by the complete conversion of form I into form II DNA (Figure 24, lanes b) (Naseem et al., 1993). Figure 24A shows the inhibition of the reaction in the presence of 25 and 50 μM concentration of all the three curcuminoids. Figure 24B compares the inhibition at a 100 μM curcuminoid concentration. It is seen that all the curcuminoids protect supercoiled plasmid DNA against cleavage by $^1O_2$ to almost similar extent.

Inhibition of hydroxyl radical mediated degradation of supercoiled plasmid DNA by curcuminoids

The generation of $OH^{\cdot}$ was carried out by two methods i.e., through Fe(II)-EDTA-H_2O_2 and kojic acid-Fe(II) systems. As seen in figure 25A, supercoiled plasmid DNA is degraded by Fe(II)-EDTA in the presence of H_2O_2 and ascorbate. The curcuminoids used have a protective effect with all the three showing similar degree of inhibition of cleavage. The results with kojic acid-Fe(II) system were also found to be qualitatively similar (fig. 25B).

Figure 23 : Inhibition of riboflavin mediated singlet oxygen ($^1O_2$) production by curcumin, demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC).

The photogeneration of $^1O_2$ by riboflavin was carried out as described in Methods. The generation of $^1O_2$ was measured by recording the decrease in the absorption of pRNO solution (0.05 mM in 10 mM phosphate buffer, pH 8.0) containing 10 mM histidine as a selective acceptor of $^1O_2$. The concentration of both curcuminoids and riboflavin in the reaction mixture was 0.1 mM.

(✱) pRNO alone; (o) pRNO + riboflavin + NaN_3 (20 mM); (●) pRNO + riboflavin + curcumin; (Δ) pRNO + riboflavin + dmC; (▲) pRNO + riboflavin + bdmC; (□) pRNO + riboflavin.

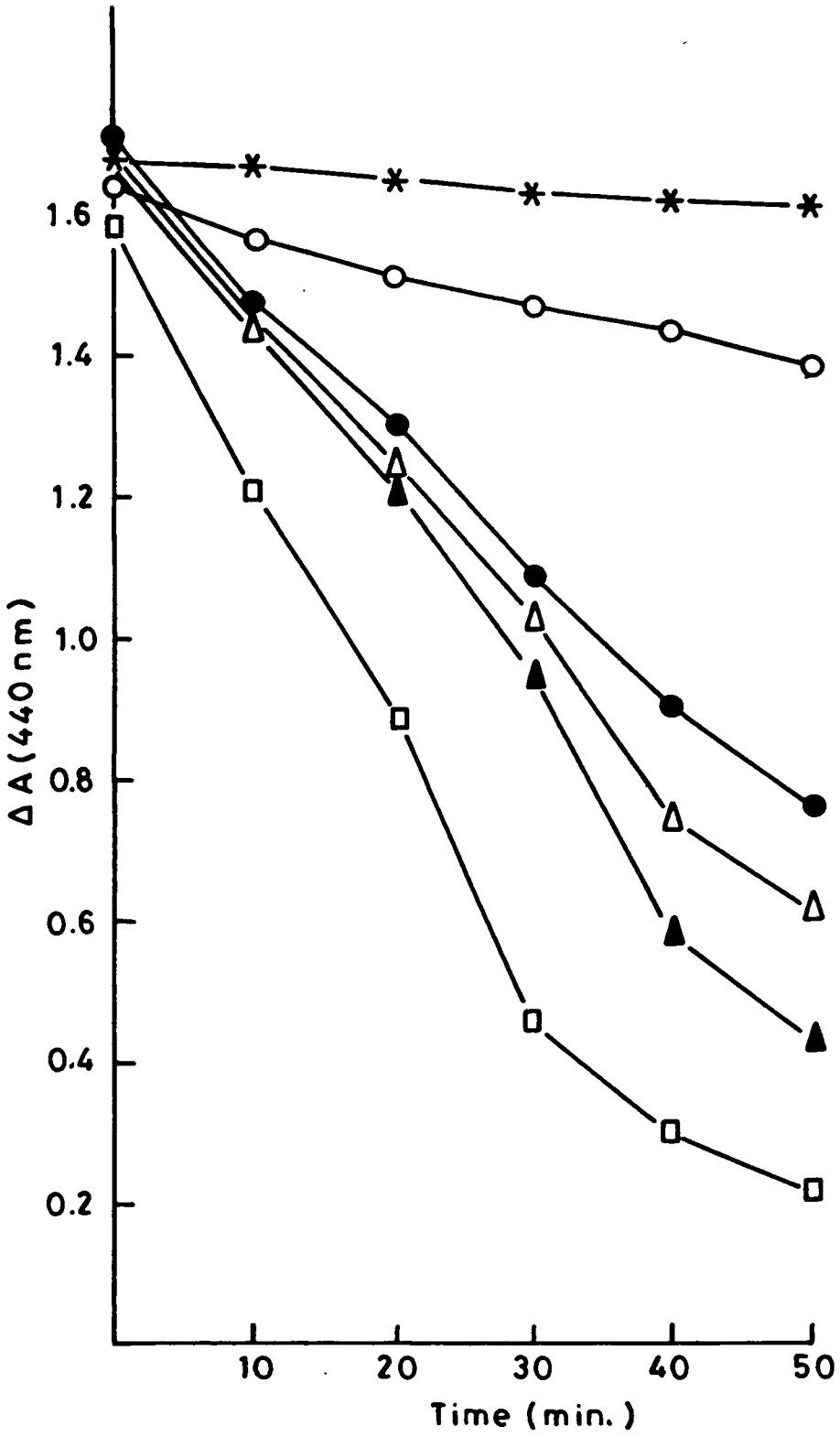

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
ΔA (440 nm)
10
20
30
40
50
Time (min.)

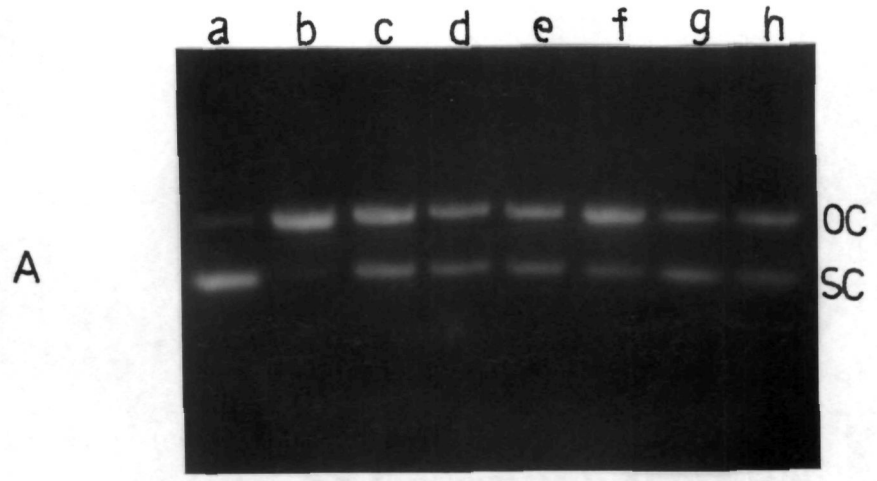
Figure 24 : **Inhibition of singlet oxygen induced cleavage of plasmid DNA by curcumin, demethoxycurcumin (dmC) and bisdemethoxy-curcumin (bdmC).**

A : Supercoiled plasmid pBR322 DNA (0.5 μg) was treated with 0.1 mM riboflavin and curcuminoids for 1.5 hours at room temperature in the presence of light.

Lane (a) DNA alone, (b) DNA + riboflavin, (c) DNA + riboflavin + curcumin (25 μM), (d) DNA + riboflavin + curcumin (50 μM), (e) DNA + riboflavin + dmC (25 μM), (f) DNA + riboflavin + dmC (50 μM), (g) DNA + riboflavin + bdmC (25 μM), (h) DNA + riboflavin + bdmC (50 μM).

B : Supercoiled plasmid pBR322 DNA (0.9 μg) was treated with 0.1 mM riboflavin and 0.1 mM curcuminoids for 1 hour at room temperature in the presence of light.

Lane (a) DNA alone, (b) DNA + riboflavin, (c) DNA + riboflavin + curcumin, (d) DNA + riboflavin + dmC, (e) DNA + riboflavin + bdmC.



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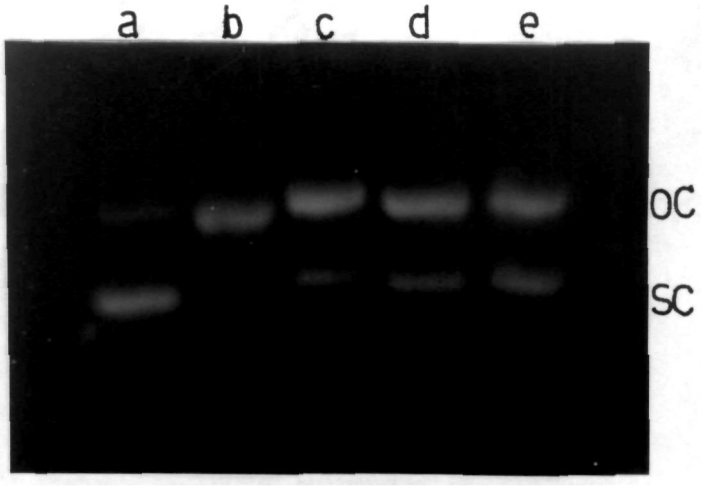

B

Figure 25A : Inhibition of hydroxyl radical [generated by Fe(II)-EDTA-H_2O_2] mediated degradation of plasmid DNA by curcumin, demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC).

Supercoiled plasmid pBR322 DNA (0.9 μg) was treated with 1 mM sodium ascorbate (pH 7.0), 40 μM Fe(II), 80 μM EDTA and 0.03% H_2O_2 in the presence of 0.2 mM curcuminoids for 10 minutes at room temperature.

Lane (a) DNA alone, (b) DNA + ascorbate + Fe(II)-EDTA + H_2O_2, (c) DNA + ascorbate + Fe(II)-EDTA+ H_2O_2 + curcumin, (d) DNA + ascorbate + Fe(II)-EDTA + H_2O_2 + dmC, (e) DNA + ascorbate + Fe(II)-EDTA + H_2O_2 + bdmC.

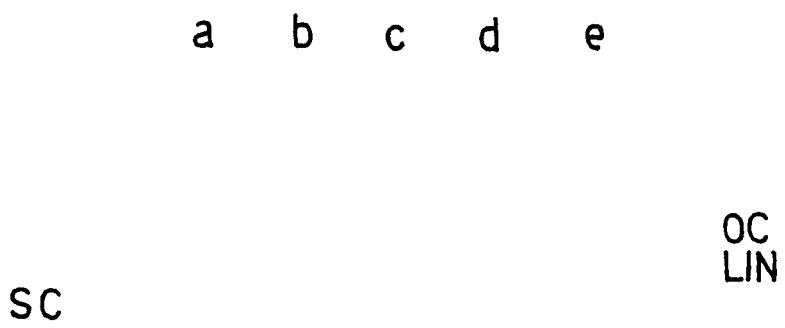
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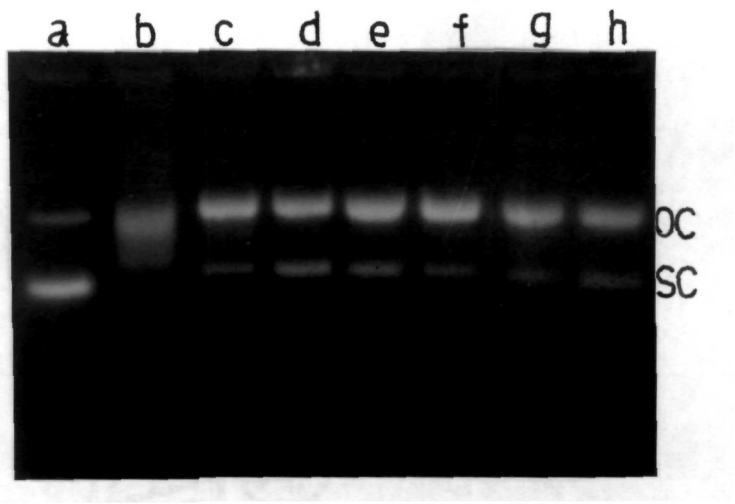
Figure 25B : Inhibition of hydroxyl radical [generated by Kojic acid-Fe(II)] mediated degradation of plasmid DNA by curcumin, demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC).

Supercoiled plasmid pBR322 DNA (0.7 μg) was treated with 50 μM kojic acid and 50 μM Fe(II) in the presence of 0.1 mM and 0.2 mM curcuminoids for 1.5 hours at room temperature.

Lane (a) DNA alone, (b) DNA + kojic acid + Fe(II), (c) DNA + kojic acid + Fe(II) + curcumin (0.1 mM), (d) DNA + kojic acid + Fe(II) + curcumin (0.2 mM), (e) DNA + kojic acid + Fe(II) + dmC (0.1 mM), (f) DNA + kojic acid + Fe(II) + dmC (0.2 mM), (g) DNA + kojic acid + Fe(II) + bdmc (0.1 mM), (h) DNA + kojic acid + Fe(II) + bdmC (0.2 mM).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Restriction analysis of curcuminoid treated lambda phage DNA

A structural change in the recognition sequence of a restriction endonuclease through covalent modification, adduct formation or strand breakage would result in the inhibition of cleavage at that site (Goppelt et al., 1981). Thus restriction endonucleases can be used in the studies on structural alteration of DNA caused by DNA specific drugs. I have used four restriction enzymes namely EcoRI, SmaI, BamHI and HindIII to evaluate curcuminoid-DNA interaction. These enzymes have exclusively consecutive AT or GC base pairs in their recognition sequences. Figure 26 shows an experiment where lambda phage DNA was treated with curcuminoids and digested with EcoRI (G/AATTC). It is seen that at 100 and 200 μM curcumin concentrations, there is partial digestion of reacted DNA (lanes b and c). However, at both the above concentrations, complete inhibition of cleavage by EcoRI is observed for dmC and bdmC. A similar experiment was performed using the EcoRI* activity of EcoRI. It is known that under the conditions of low ionic strength, high enzyme concentration and high pH, a reduction occurs in the specificity of the hexanucleotide recognition sequence of EcoRI to a tetranucleotide sequence (/AATT) (Polisky et al., 1975). Thus the EcoRI* recognition sequence contains exclusively AT base pairs. Figure 27(A) shows that at 25 and 50 μM curcumin concentration, only partial digestion of treated DNA occurs (lanes b and c). However, at higher concentrations of 100 and 200 μM (lanes d and e) EcoRI*

Figure 26 : EcoRI digestion of curcumin, demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC) treated lambda-phage DNA.

Lambda DNA (2 μg) was treated with 100 μM (lanes b, c and d) and 200 μM (lanes e, f and g) concentration of curcuminoids before dialysis and digestion with EcoRI.

Lane (a) is a control untreated sample. Lanes (b) and (e) 100 and 200 μM curcumin; (c) and (f) 100 and 200 μM dmC and (d) and (g) 100 and 200 μM bdmC.

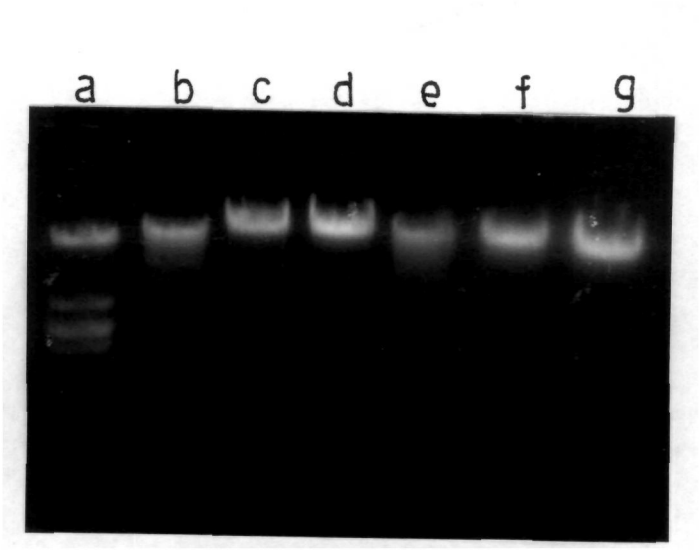
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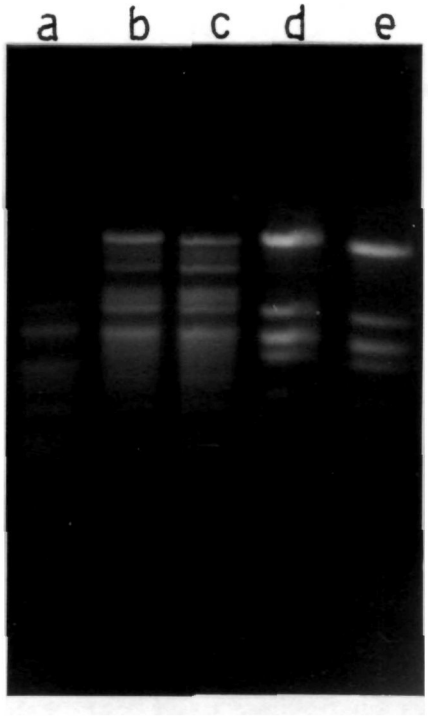
Figure 27A : EcoRI* digestion of curcumin treated lambda-phage DNA.

Lambda DNA (1 μg) was treated with 25, 50, 100 and 200 μM concentrations of curcumin (lanes b, c, d and e respectively) before dialysis and digestion with EcoRI*. Lane (a) is a control untreated sample.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cleavage pattern is not seen and the pattern appears to revert back to EcoRI cleavage. With dmC (Fig. 27B) no inhibition occurs at 25 μM concentration, partial inhibition at 50 and 100 μM concentrations, while at 200 μM EcoRI cleavage pattern appears. In the case of bdmC (Fig. 27C), no inhibition is seen at 25 and 50 μM concentrations, partial inhibition at 100 μM and the EcoRI cleavage pattern is observed at 200 μM bdmC concentration. Figure 28 gives an experiment showing EcoRI* activity with all the three compounds showing complete or partial abolition of EcoRI* cleavage pattern.

The experiments shown in figure 29, was performed using SmaI whose recognition sequence contains exclusively GC base pairs (CCC/GGG). With the concentration of all three curcuminoids used (0.05 and 0.1 mM) no inhibition of cleavage was observed with SmaI. The results with EcoRI, EcoRI* and SmaI indicate that curcuminoids preferentially bind to AT base pairs. In order to substantiate this observation, two further experiments were performed, one with BamHI (G/GATCC) containing GC base pairs flanking an AT base pair and the other with HindIII (A/AGCTT) containing AT base pairs flanking a GC base pair. With BamHI (Fig. 30), no inhibition of cleavage is seen for any of the curcuminoids, while HindIII is inhibited to various degrees by the curcuminoids (Fig. 31). The results further confirm that curcuminoids bind to AT base pairs in a way that leads to the inhibition of cleavage by the appropriate restriction endonuclease.

Figure 27B : EcoRI* digestion of demethoxycurcumin (dmC) treated lambda-phage DNA.

Lambda DNA (1 μg) was treated with 25, 50, 100 and 200 μM concentrations of dmC (lanes b, c, d and e respectively) before dialysis and digestion with EcoRI*. Lane (a) is a control untreated sample.

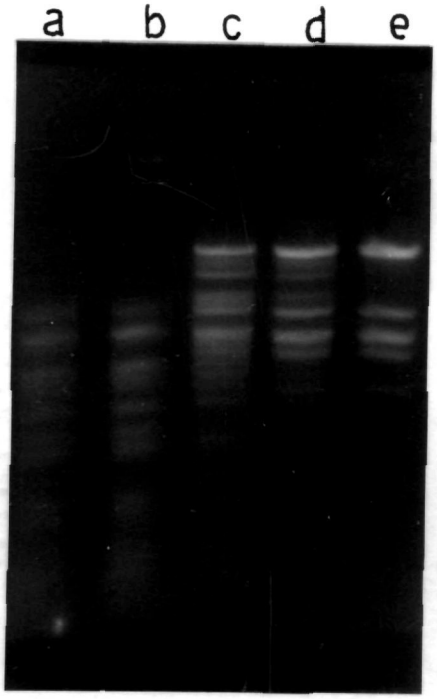
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Figure 27C : EcoRI* digestion of bisdemethoxycurcumin (bdmC) treated lambda-phage DNA.

Lambda DNA (1 μg) was treated with 25, 50, 100 and 200 μM concentrations of bdmC (lanes b, c, d and e respectively) before dialysis and digestion with EcoRI*. Lane (a) is a control untreated sample.

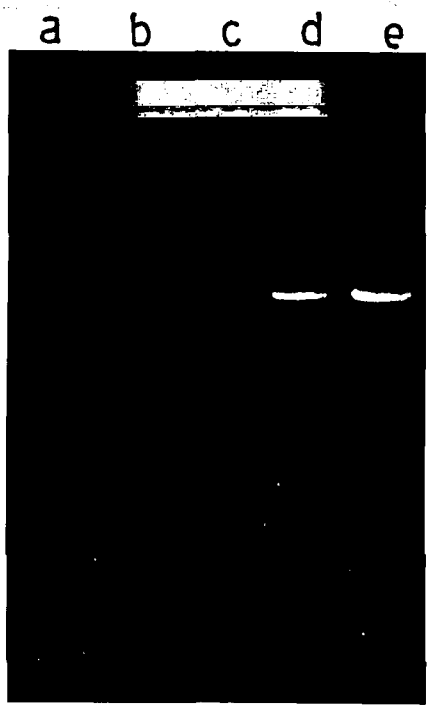
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Figure 28 : EcoRI* digestion of curcumin, demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC) treated lambda-phage DNA.

Lambda DNA (2 μg) was treated with 25 μM (lanes b, c and d) and 50 μM (lanes e, f and g) concentration of curcuminoids before dialysis and digestion with EcoRI*.

Lane (a) is a control untreated sample. Lanes (b) and (e) 25 and 50 μM curcumin; (c) and (f) 25 and 50 μM dmC and (d) and (g) 25 and 50 μM bdmC.

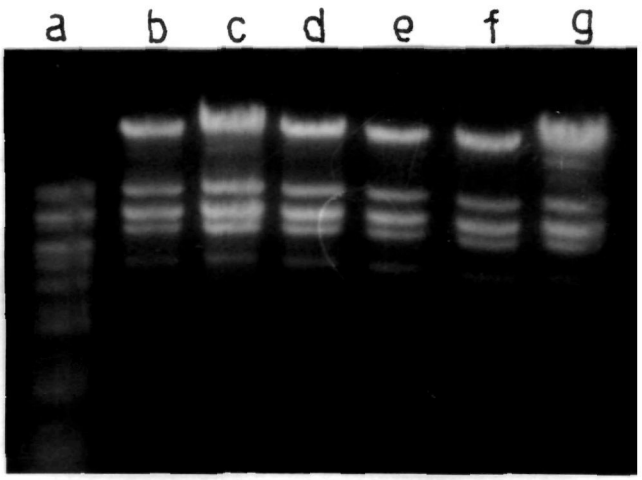
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Figure 29 : SmaI digestion of curcumin, demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC) treated lambda-phage DNA.

Lambda DNA (1 μg) was treated with 50 μM (lanes b, c and d) and 100 μM (lanes e, f and g) concentration of curcuminoids before dialysis and digestion with SmaI.

Lane (a) is a control untreated sample. Lanes (b) and (e) 50 and 100 μM curcumin; (c) and (f) 50 and 100 μM dmC and (d) and (g) 50 and 100 μM bdmC.

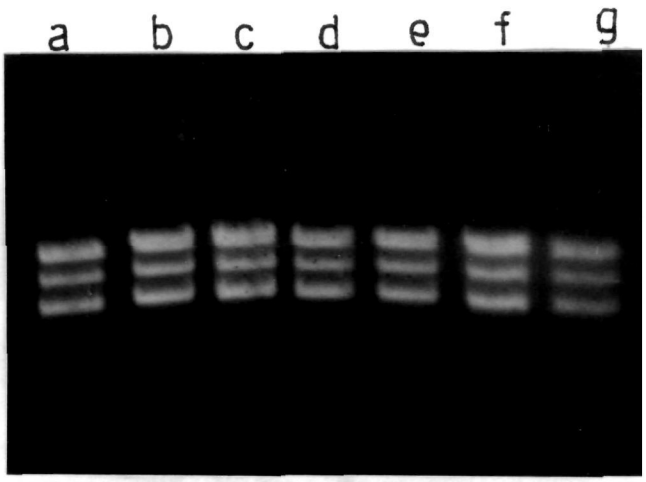
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Figure 30: BamHI digestion of curcumin, demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC) treated lambda-phage DNA.

Lambda DNA (2 μg) was treated with 50 μM (lanes b, c and d) and 100 μM (lanes e, f and g) concentration of curcuminoids before dialysis and digestion with BamHI.

Lane (a) is a control untreated sample. Lanes (b) and (e) 50 and 100 μM curcumin; (c) and (f) 50 and 100 μM dmC and (d) and (g) 50 and 100 μM bdmC.

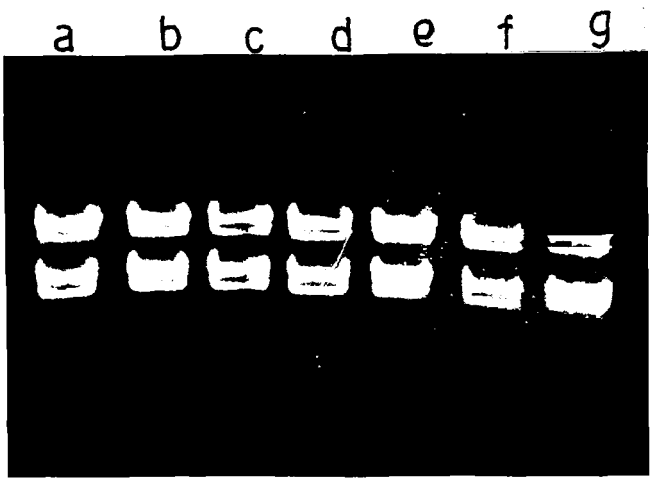
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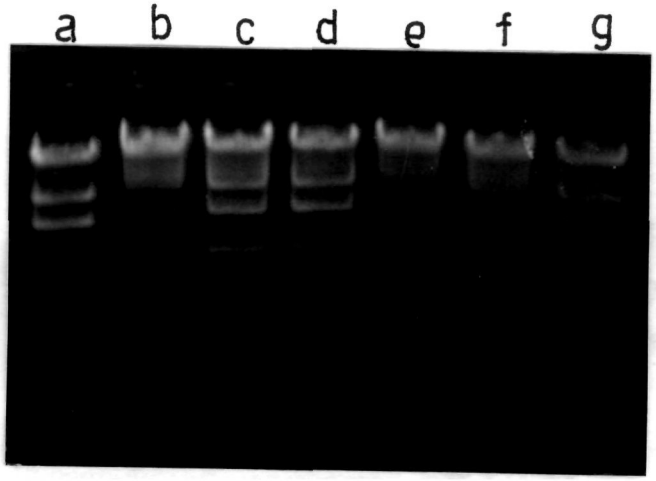
Figure 31 : **HindIII digestion of curcumin, demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC) treated lambda-phage DNA.**

Lambda DNA (2 μg) was treated with 50 μM (lanes b, c and d) and 100 μM (lanes e, f and g) concentration of curcuminoids before dialysis and digestion with HindIII.

Lane (a) is a control untreated sample. Lanes (b) and (e) 50 and 100 μM curcumin; (c) and (f) 50 and 100 μM dmC and (d) and (g) 50 and 100 μM bdmC.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DISCUSSION
(PART II)

DISCUSSION (Part II)

The studies presented above lead to the following major conclusions :

i) Curcumin and its derivative curcuminoids, dmC and bdmC cleave supercoiled plasmid DNA in the presence of Cu(II), ii) Curcuminoids generate reactive oxygen species mainly hydroxyl radicals and hydrogen peroxide, iii) Curcuminoids exhibit characteristic absorption spectral changes in the presence of DNA and Cu(II) suggesting binding to both these substrates, iv) Curcuminoids are capable of inhibiting damage to supercoiled plasmid DNA by singlet oxygen and hydroxyl radicals. They are also effective quenchers of singlet oxygen generated by photoilluminated riboflavin, and finally v) Curcuminoids bind preferentially with sequences of AT base pairs.

A number of studies have been carried out comparing radical scavenging and antioxidant properties of curcumin with its two closely related derivatives demethoxycurcumin (dmC) and bisdemethoxycurcumin (bdmC). Sreejayan and Rao (1996 and 1994) in two separate studies have compared them for their free radical scavenging activity and also their effect on iron-catalysed lipid peroxidation, respectively. In the latter study it was found that all the three compounds were equally active but more potent than α-tocopherol as inhibitors of iron-catalysed lipid peroxidation in rat brain homogenates and liver microsomes. The three curcuminoids were also compared for their ability to scavenge superoxide radicals (Sreejayan and Rao, 1996). It was shown that

curcumin is the most effective scavenger of superoxide radicals followed by dmC and bdmC. An earlier study by Subramanian et al., (1994) have established that curcumin and its derivatives are capable of protecting plasmid DNA against singlet oxygen induced strand breaks. Curcumin was found to be the most effective inhibitor of DNA damage followed by dmC and then bdmC.

It is interesting to note from the present results that as a DNA damaging pro-oxidant in the presence of Cu(II), curcumin was found to be the most effective followed by dmC and bdmC. Thus both the antioxidant as well as pro-oxidant effects of curcuminoids follow the same pattern. It therefore appears that the structural features of curcuminoids that are important for their antioxidant effects are also the ones that render these compounds DNA damaging under appropriate conditions. This indicates that the presence of methoxy groups in curcumin is an important structural feature for the biological properties of curcuminoids.

Biological antioxidants show great variation in their modulation of DNA damage induced by oxygen free radicals. Thiols which protect DNA as well as other biomolecules against $OH^{\cdot}$ and ionising radiation do not protect it against O_2^{-} (Sies, 1989). For example, glutathione, cysteine and dithiothreitol significantly enhance DNA damage induced by O_2^{-}. However, lipid soluble antioxidants such as carotenoids, butein, tannic acid offer significant protection to plasmid pBR322 DNA against O_2^{-} (Sah et al., 1995). Many plant phenolics especially flavonoids have been described as antioxidants because they inhibit

lipid peroxidation. However, several plant phenolics can accelerate oxidative damage to biomolecules such as DNA (Laughton et al., 1989). They do this by reducing Fe(III) ions to Fe(II) or by producing O_2^- and H_2O_2 through oxidation. Phenols have complex pro- and antioxidant effects in vitro depending on the assay system used, and is often hard to predict their net effect in vivo (Halliwell, 1990). For example, many synthetic and dietary polyphenols such as quercetin, catechin, gallic acid, caffeic acid can protect cells from the cytotoxicity induced by hydrogen peroxide (Nakayama, 1994). Phenolic compounds that cause DNA damage in the presence of copper ions include BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), dopamine, ferulic acid, caffeic acid, flavonoids, tannic acid, etc. (Bhat and Hadi, 1994; Li et al., 1994; Li and Trush, 1994; Ahmad et al., 1992). This interaction could cause a range of DNA lesions including base modifications, strand breaks and phenol adducts to the DNA bases, all of which might contribute to the carcinogenecity of certain phenolic compounds (Wiseman and Halliwell, 1996). Thus, an antioxidant in one system is not an antioxidant in all systems.

Copper is absorbed in the anterior portion of the intestine and is stored mainly in the liver. Most of the serum copper is bound to ceruloplasmin. The concentration of copper in blood serum is under homeostatic control. However, an increase in plasma copper or copper binding protein ceruloplasmin titre has been recorded during second and third trimester of pregnancy in humans. The elevated amount of plasma copper in women on oral contraceptive agents or in

post-menopausal women with estrogen replacement is also reflected as increased plasma ceruloplasmin level (Sharma and Sharma, 1997).

Copper is capable of mediating activation of a variety of phenolic compounds, producing reactive oxygen and electrophilic phenolic intermediates (Li and Trush, 1994). Copper is an essential trace element which is distributed throughout the body. It is present in many tissues including liver and kidney where its concentration is relatively high (Linder, 1991). Besides forming the essential redox-active centre in a variety of metalloproteins such as ceruloplasmin, Cu/Zn superoxide dismutase, cytochrome c oxidase, tyrosinase, ascorbate oxidase, etc., copper has also been found in the nucleus and to be closely associated with chromosomes and DNA bases, particularly guanine (Kagawa et al., 1991; Agarwal et al., 1989). The interaction of phenolic compounds with DNA-associated copper may result in a spectrum of DNA lesions including oxidative DNA base modification, strand breaks and DNA adducts of phenolic intermediates (Li and Trush, 1994).

A majority of the antioxidants present in plants or added to foods contain phenolic structures and their potencies are frequently tested only in lipid systems. Phenolic antioxidants such as propyl gallate, gossypol, carnosic acid, carnosol are capable of stimulating free radical damage to non-lipid components, carbohy-drates and DNA in vitro. It is therefore important to consider the potential pro-oxidant actions of food additives and nutrient components (Aruoma, 1993).

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PRESENTATIONS/PUBLICATIONS

1. Haseeb Ahsan, Nasir K. Alvi and S.M. Hadi, 'Strand scission in DNA induced by dietary curcumin and Cu(II).' Presented at the 64th Annual Meeting of Society of Biological Chemists (India), held at Lucknow from 6-8 Oct., 1995.

2. Haseeb Ahsan and S.M. Hadi, 'Generation of reactive oxygen species by curcumin.' Presented at the 65th Annual Meeting of Society of Biological Chemists (India), held at Bangalore from 20-23 Nov., 1996.

3. Haseeb Ahsan and S.M. Hadi, 'Sequence preference in the binding of curcuminoids to DNA.' Presented at the 66th Annual Meeting of Society of Biological Chemists (India), held at Visakhapatnam from 22-24 Dec., 1997.

4. Haseeb Ahsan and S.M. Hadi, 'Strand scission in DNA induced by curcumin in the presence of Cu(II).' Cancer Letters (in press).